Characterization of Active Cellulolytic Consortia from Arctic Tundra

by

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AUTHOR'S DECLARATION

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.

ABSTRACT

The consortia of microorganisms responsible for the hydrolysis of cellulose *in situ* are at present poorly characterized. Nonetheless, the importance of these communities is underscored by their capacity for converting biomass to greenhouse gases such as carbon dioxide and methane. The metabolic capacities of these organisms is particularly alarming considering the volume of biomass that is projected to re-enter the carbon cycle in Arctic tundra soil environments as a result of a warming climate. Novel cold-adapted cellulase enzymes also present enormous opportunities for a broad range of industries. DNA stable-isotope probing (DNA-SIP) is a powerful tool for linking the phylogenetic identity and function of cellulolytic microorganisms by the incorporation of isotopically labelled substrate into nucleic acids. By providing ^{13}C-enriched glucose and cellulose to soil microcosms, it was possible to characterize the communities of microorganisms involved in the metabolism of these substrates in an Arctic tundra soil sample from Resolute Bay, Canada. A protocol for generating ^{13}C-enriched cellulose was developed as part of this thesis, and a visual DNA-SIP protocol was generated to demonstrate the experimental outline. Denaturing gradient gel electrophoresis (DGGE) and 16S rRNA clone libraries were used to visualize changes in community structure and to identify prevalent, active phylotypes in the SIP incubations. Notably, predominant phylotypes changed over time and clustered based on substrate metabolism. Labelled nucleic acids identified by sequenced DGGE bands and 16S rRNA gene clone libraries provided converging evidence indicating the predominance of *Clostridium* and *Sporolactobacillus* in the ^{13}C-glucose microcosms, and *Betaproteobacteria*, *Bacteroidetes*, and *Gammaproteobacteria* in the ^{13}C-cellulose microcosms. Active populations consuming glucose and cellulose were distinct based on principle coordinate analysis of “light” and “heavy” DNA. A large portion of the recovered

sequences possessed no close matches in the GenBank database, reflecting the paucity of data on these communities of microorganisms.

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LIST OF ABBREVIATIONS

ARISA	automated ribosomal intergenic spacer analysis
ATCC	American Type Culture Collection
Bp	base pair(s)
BLAST	Basic Local Alignment Search Tool
C	carbon atom
cm	centimetre(s)
CsCl	cesium chloride
d	day(s)
ddH_2O	distilled and deionized water
DGGE	denaturing gradient gel electrophoresis
DNA	deoxyribonucleic acid
DNA-SIP	deoxyribonucleic acid stable-isotope probing
dNTP	deoxyribonucleoside triphosphate
EDTA	ethylenediamine tetraacetate
EtBr	ethidium bromide
g	gram(s)
g_{av}	average gravity
g cm^{-3}	grams per cubic centimetre
g L^{-1}	grams per litre
g mL^{-1}	grams per millilitre
G+C	guanine-cytosine content
HCl	hydrogen chloride
Kb	kilobase pair(s)
KCCM	Korean Culture Centre of Microorganisms
Kg	kilogram
KiNG	Kinemage Next Generation
km^2	square kilometres
L	litre(s)
M	molar
mg	milligram(s)
mg ml^{-1}	milligrams per millilitre
min	minute(s)
mL	millilitre(s)
mm	millimetre(s)
mM	millimolar
mmoX	methane monooxygenase
m s^{-1}	metres per second
mxaF	methanol dehydrogenase
N	nitrogen atom
ng	nanogram(s)
nmol	nanomolar
NaCl	sodium chloride
O	oxygen atom
PCoA	principle coordinates analysis
PCR	polymerase chain reaction
PEG	polyethylene glycol

Pg	petagram
PLFA-SIP	phospholipid fatty acid stable-isotope probing
pmoA	particular methane monooxygenase gene
QIIME	quantitative insights into microbial ecology
RDP	Ribosomal Database Project
RNA-SIP	ribonucleic acid stable-isotope probing
rpm	revolutions per minute
rRNA	ribosomal ribonucleic acid
s	second(s)
spp.	species
TE	10 mM Tris HCl (pH 8.0), 1 mM EDTA
T-RFLP	terminal restriction fragment length polymorphism
Tris	tris (hydroxymethyl) amino methane
µg	microgram(s)
µg g^{-1}	micrograms per gram
µl	microlitre(s)
µl min^{-1}	microlitres per minute
µm	micrometre(s)
µmol	micromolar
UniFrac	unique fraction metric
UV	ultraviolet
V	volts
w/v	weight per volume

Chapter 1 Literature Overview

1.1 Cellulolysis

1.1.1 Introduction to cellulose

Cellulose is a linear homopolysaccharide consisting of anhydroglucose subunits linked by beta 1,4-glycosidic bonds (Kleman-Leyer et al., 1996; Koizumi et al., 2008). Cellulose is found within nearly every Earth ecosystem as a component of the cell walls of plants and algae, and is also the most abundant waste product generated by human society (Bayer et al., 2007). Cellulose represents the main structural unit of plant cell walls, accounting for 35-50% of biomass dry weight (Lynd et al., 2002; Schellenberger et al., 2010). Consequently, it is the most abundant organic component of the biosphere (Lynd, et al., 2002; O'Sullivan, et al., 2007; Ross et al., 1991). Cellulose is readily obtained from waste materials such as sewage and unused agricultural biomass. Availability and a plentiful supply make cellulose a realistic material to be exploited as a renewable energy source (Lynd et al., 2002).

Cellulolysis is the process by which cellulose is decomposed. Hydrolysis of cellulose is a multi-stage process involving many intermediate steps. Cellulose can be broken down both aerobically and anaerobically, and therefore there are two general metabolic pathways for the bioconversion of cellulose. Anaerobic degradation typically comprises a complex system of hydrolysis due to the presence of external protein complexes called cellulosomes, which are associated with the cell walls of anaerobic cellulolytic microorganisms (Lynd et al., 2002). Cellulosomes contain a suite of enzymes that allow for maximal cellulose degradation in close

proximity to the host organism (Doi & Kosugi, 2004). In anaerobic environments, cellulolytic microorganisms such as clostridia cleave cellulose fibres into cellobiose, glucose, and other cellodextrins (Figure 1.1). These molecules are then converted to propionate, butyrate, acetate, formate, hydrogen gas (H_2), and carbon dioxide (CO_2) by cellulolytic and fermentative organisms. Syntrophs and homoacetogens can also utilize some of these substrates. Methanogens convert the remaining acetate, formate, H_2, and CO_2 to methane (CH_4).

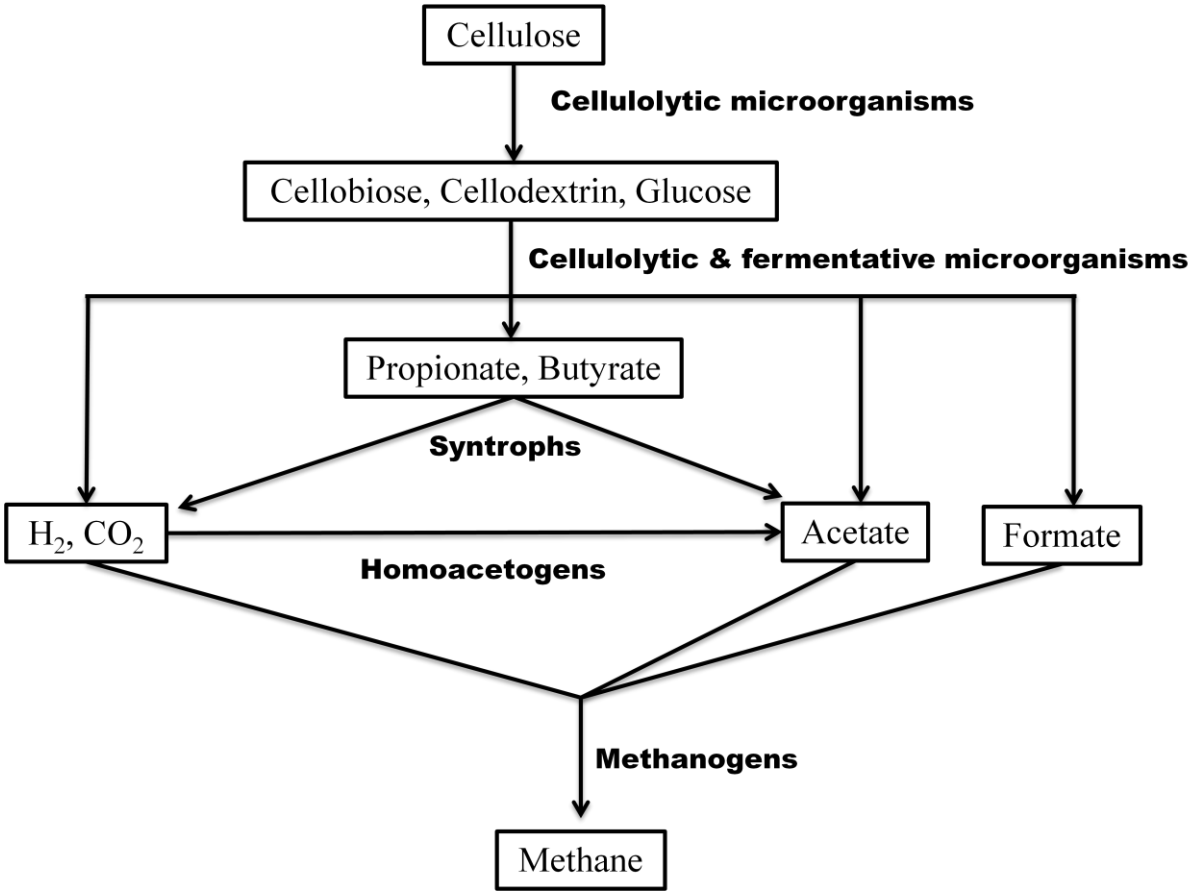

Figure 1.1 Anaerobic cellulose degradation by microbial communities. Modified from Leschine, (1995)

Under aerobic conditions, cellulosic polymers are hydrolyzed to sugar monomers by extracellular cellulase enzymes. Cellulose hydrolysis enzymes produced by microorganisms have been divided into three major classes: exoglucanases that operate at the non-reducing end

of cellulose, releasing either cellobiose or glucose, endoglucanases that randomly cleave the cellulose chain by splitting beta-1-4 glucosidic linkages, and cellobiases that hydrolyse cellobiose and other cellodextrins to glucose monomers. Collectively, this diverse group of enzymes are referred to as "cellulases", which are part of the glycosyl hydrolase group of enzymes (Doi & Kosugi, 2004). These extracellular enzymes are typically produced in excess, and possess unique modules for binding to different conformations of cellulose. Cellulases produced by the host organism act in concert to completely hydrolyze cellulose (Lynd et al, 2002).

The aerobic model of cellulose hydrolysis is largely based on 50 years of research into the cellulase system of the aerobic filamentous fungus *Trichoderma reesei* (Lynd et al., 2002; Zhang & Lynd, 2004). *T. reesei* produces a mix of extracellular enzymes including two cellobiohydrolases, five endoglucanases, β-glucosidases, and hemicellulases. These different enzymes can function synergistically to achieve a maximal rate of degradation. Exoglucanases attack amorphous (non-organized chains of cellulose susceptible to degradation) regions of cellulose, exposing the sites for cellobiohydrolases which can hydrolyze crystalline (structured chains of cellulose that are difficult to degrade) regions (Pérez et al., 2002). β-glucosidases reduce cellobiose produced by the cellobiohydrolases to glucose, completing the hydrolysis reaction (Beguin, 1990; Zhang & Lynd, 2004).

1.1.2 Cellulolysis: challenges

As described above, the bioconversion of cellulose is a multi-stage enzymatic process carried out by extracellular cellulase enzymes that are either free or cell-associated (Li et al., 2009). Although many organisms can metabolize the base glucose unit of cellulose, the structure of the overall polymer within the plant presents a challenge for degradation. The ability to digest cellulose is restricted to microorganisms in the domains *Bacteria* and *Eucarya*; at present no cellulolytic *Archaea* have been identified (Lynd et al., 2002; el Zahar Haichar et al., 2007).

The capture of light energy during plant photosynthesis provides an energy storage method in the form of cell wall polymers (Rubin, 2008). Cellulose is generated by plants as individual molecules that combine to form densely structured cellulose fibres. The arrangement of the cellulose molecules creates strong intrachain and interchain hydrogen bonds. The crystalline structure of plant cellulose makes it extremely resistant to enzymatic hydrolysis (Doi & Kosugi, 2004; Lynd et al., 2002). However, cellulose fibrils possess amorphous domains that make it partially soluble in water, and the abundance of these domains is dependent on the organism that produced the fibril (Koizumi et al., 2008). Cellulose fibres also possess a variety of structural irregularities, including twists, pores, and capillaries. Immersion in water can thus provide opportunities for cellulase activity, as saturated pores can expose sections of the fibre to cellulolytic enzymes (Stone & Scallan, 1968; Stone et al., 1969). The structural complexity and heterogeneity of cellulose fibril structure makes cellulose a recalcitrant substrate for microorganisms to degrade (Kleman-Leyer et al., 1996; Lynd et al., 2002). Enzymatic degradation occurs outside of the host organism, as necessitated by the relatively insoluble

nature of the cellulose substrate. Cellulases are either found in association with the cell wall complex or persist exocellularly (Leschine, 1995; Schwarz, 2001).

Additionally, cellulose polymers in plant material are intertwined with other structural molecules such as hemicelluloses, pectin, and lignin (Bayer et al., 2007). For most plant cell walls, cellulose fibres account for only 15-40% of the overall structure. Approximately 30-40% is hemicelluloses and pectin, with lignin accounting for the remaining 20% (Doi & Kosugi, 2004). Although hemicellulose, pectin, and lignin are easier to degrade in comparison to pure cellulose, their presence in natural cellulosic material provides a complex structure that further constrains the activity of cellulolytic enzymes (Lynd et al., 2002; Wilson & Mertens, 1995).

Due to the rigid structure of cellulose, and the varied structure of plant material, a suite of enzymes is often required for complete metabolism of the substrate. Based on extensive research of the cellulase system of the fungus *T. reesei*, a synergistic model of aerobic enzymatic degradation of cellulose has been accepted for some time (Schwarz, 2001). Recently, it has been suggested that this synergistic, three-class model is an oversimplification of a much more complex process (Schwarz, 2001). Maximal cellulose degradation is achieved by mixtures of different enzymes, indicating that degradation by different enzymes from a variety of different microorganisms is much more efficient (Haruta et al., 2002; Singh & Hayashi, 1995).

Cellulolytic microorganisms have also been shown to form commensal relationships with other microorganisms that are not directly involved in cellulolysis. The complete breakdown of cellulose is typically a function of the entire community, and not just the microorganisms that are directly involved (Beguin & Aubert, 1994; Leschine, 1995). For example, the contributions of

carbohydrate and hydrogen consumers enhance the speed and efficiency of cellulose hydrolysis in mixed cultures (Kato et al., 2004). This enhancement is not surprising considering that most of the enzymes involved in cellulolysis are exocellular. The products of cellulolysis are available to other microorganisms in the community as carbon and energy sources (Leschine, 1995). In turn, the activities of these other microorganisms are likely to affect the cellulose-degrading organisms (Haruta et al., 2002). For example, Pohlschroeder et al., (1994) reported that thermophilic *Spirochaeta caldaria* were able to form stable co-cultures with anaerobic cellulose-degrading Clostridia. It is likely that there are many trophic interactions between microorganisms or "consortia" in the cellulolytic community.

1.1.3 Cellulolytic organisms and communities

Until recently, research on cellulolytic bacterial microorganisms has focused on a select number of well-characterized model organisms. The vast majority of these characterized microorganisms are anaerobic mesophiles or thermophiles (Doi & Kosugi, 2004). Degradation of cellulose can occur in either aerobic or anaerobic environments. Most cellulose is degraded aerobically, with approximately 5-10% degraded anaerobically (Pérez et al., 2002). As of 2001, more than 600 cellulase genes have been identified (Schwarz, 2001). These enzymes work in concert to synergistically degrade the substrate. Because the assembly of the enzyme systems occurs exocellularly (Leschine, 1995), it is likely that enzymes from multiple species can interact to enhance cellulolysis.

The synergistic model of *T. reesei* is representative of typical aerobic degradation. Other fungal genera have been identified as aerobic degraders of cellulose including members of the

Ascomycota and *Basidiomycota* (Dowson et al., 1988), in addition to bacterial genera such as *Bacillus* (low G+C *Firmicutes*), *Streptomyces* (high G+C *Actinomycete*), *Pseudomonas*, *Cytophaga*, *Cellvibrio*, and *Cellulomonas* (Hulcher & King, 1958; Lynd et al., 2002).

Organisms adapted to anaerobic conditions degrade cellulose in a completely different manner. *Clostridium thermocellum* has become a model organism for anaerobic cellulase activity due to its potential for cellulose degradation. A wealth of data on *C. thermocellum* exists in the literature as a result of its proficiency for cellulose degradation (Bayer et al., 1985; Hulcher & King, 1958; Lamed et al., 1983; Zverlov & Schwarz, 2008). *Clostridium thermocellum* is ubiquitous in soil environments and is capable of generating a cellulosome – a suite of extracellular proteins dedicated to maximal degradation of cellodextrins (Zverlov & Schwarz, 2008).

The term "cellulosome" was coined to describe the hydrolytic strategy of anaerobic microorganisms such as *Clostridium* spp. for cellulose degradation. The cellulosome is an extracellular multi-enzyme complex that binds cells directly to crystalline cellulose particles (Schwarz, 2001). Each cellulosome contains a "scaffoldin" protein which tethers the cell to the substrate via a cellulose-binding module (Bayer et al., 2007; Doi & Kosugi, 2004). The cellulosome complex can incorporate up to nine additional enzymes in addition to the scaffoldin protein. Cellulases, endoglucanases, xylanases, and cellobiohydrolases are all common catalytic components that are employed in hydrolysis (Schwarz, 2001). The use of a cellulosome is thought to enhance the synergy of cellulolysis, as all the necessary enzymes are encoded within the genome of one cell.

Anaerobic degradation of cellulose by the bacterial genera *Clostridium*, *Acetivibrio*, *Ruminococcus,* and *Caldicellulosiruptor* has been well characterized (Krause et al., 2008; Lynd et al., 2002; Schwarz, 2001). Members of the fungal phylum *Chytridiomycota* have also been shown to be capable of anaerobic cellulolysis. Microbial communities engaging in anaerobic cellulolysis are typically less diverse than aerobic communities (Lynd et al., 2002). Often co-cultures of only two species are capable of anaerobic cellulolysis in laboratory settings, possibly due to the presence of the cellulosome complex (Liu et al., 2008). It is extremely common for *Firmicutes* (particularly *Clostridium* spp.) to dominate in anaerobic environments (Krause et al., 2008). It is suspected, however, that the full complement of bacteria, fungi, and archaea that are capable of performing cellulolysis in terrestrial environments has yet to be identified, with evidence indicating that many or most organisms involved are currently uncultivated (de Boer et al., 2005).

1.1.4 Recent advances in the understanding of cellulolytic communities *in situ*

The well-documented cellulose-degrading ability of *C. thermocellum* and *T. reesei* has made these organisms the focus of intensive research (Liu et al., 2008; Lynd et al., 2002; Zhang & Lynd, 2004). The advent of the biotechnology revolution in the late 1980s and 1990s led to extensive efforts to identify and characterize the cellulose and lignin degrading systems in known cellulolytic organisms. At this point, most of the known cellulase enzymes have been successfully cloned, sequenced, and expressed in host vectors (Pérez et al., 2002).

The focus on the anaerobic thermophiles has obscured the possibility that uncultivated organisms still exist in the natural environment that could provide unique and more industrially

beneficial enzymes. Evidence also suggests that mixed cultures of microorganisms are more efficient than monocultures (Kato et al., 2004). The accessory organisms in these cultures are often representatives of uncultured bacterial species, and therefore little is known about their activity and metabolism within the cellulolytic consortia. This illustrates the inherent limitations of our knowledge of cellulose-degrading systems. The majority of what is known about cellulolytic communities is based on the behaviour of particular type specimens that have been isolated from their natural environment (Zhang & Lynd, 2004).

Recent experiments have employed molecular techniques such as 16S rRNA gene clone libraries, DNA stable-isotope probing (DNA-SIP), automated ribosomal intergenic spacer analysis (ARISA), and denaturing gradient gel electrophoresis (DGGE) in an attempt to address the problems associated with the current mixed-culture approach for industrial applications of cellulolysis. These projects have focused on the identification and characterization of previously unknown cellulolytic microorganism communities in environmental samples.

Research undertaken by el Zahar Haichar et al., (2007), Bernard et al., (2007; 2009) and (Lee et al., 2011), has identified a broad range of previously unidentified cellulolytic organisms. These studies have all used the DNA-SIP technique and isotopically labelled substrates to characterize microbial communities involved in cellulose hydrolysis in terrestrial soil environments. Substrates used included ^{13}C-labelled bacterial cellulose from *Gluconacetobacter xylinus* (el Zahar Haichar et al., 2007), ^{13}C-labelled wheat residues (Bernard et al., 2007; 2009), and ^{13}C-labelled rice callus (Lee et al., 2011). The soils used for the Bernard and el Zahar Haichar studies were from experimental agricultural fields in France, while the soil used by Lee

and colleagues was obtained from a rice paddy in Japan. All experiments were carried out under aerobic conditions.

Each of these DNA-SIP studies revealed labelling of microorganisms with similarity to known cellulose degraders. Prominent bacterial groups in the ^{13}C-cellulose amended microcosms included uncultured *Gammaproteobacteria*, *Dyella*, *Mesorhizobium*, *Sphingomonas,* and uncultured *Deltaproteobacteria* (el Zahar Haichar et al., 2007). Bernard and colleagues identified increased representation of *Betaproteobacteria* and *Gammaproteobacteria* in microcosms amended with ^{13}C-labelled wheat (Bernard et al., 2007). Lee and colleagues reported *Flavobacteria*, *Chloroflexi*, *Bacilli*, *Clostridia*, *Sphingobacteria,* and *Gammaproteobacteria* as populations involved in the aerobic metabolism of rice callus (Lee et al., 2011). These studies revealed the diversity of bacterial populations associated with cellulolysis in soil.

The data collected in these initial cellulose DNA-SIP experiments also reinforced the assertion that soil cellulolytic communities are poorly studied. In the research performed by el Zahar Haichar et al. (2007), a number of retrieved sequences could only be classified at the phylum level, particularly those associated with the *Bacteroidetes* and *Gammaproteobacteria* groups. Several of the sequences were most closely related to uncultured organisms that are known only from nucleic acid sequences retrieved from the environment. Similarly, Bernard and colleagues (2007) report enrichment of unknown organisms belonging to the *Alphaproteobacteria*, particularly members of the genus *Kaistobacter,* of which very little is known. In a related study, the same researchers found that up to 8.3% of clone libraries generated from soils could not be classified at the class level (Bernard et al., 2009). Finally, Lee

et al. (2011) noted that the closest known relatives to sequences that they retrieved from the heavy DNA of a rice paddy soil incubation experiment were quite variable. The closest matching database hits to sequences obtained from heavy DNA were classified as members of *Actinobacteria* and *TM7*; these clone matches were derived from previous human microbiome studies. The number of unknown sequences retrieved in these experiments reflects the paucity of data on active and uncultivated cellulolytic prokaryotes in soil environments. To help overcome these limitations, further research efforts must be directed towards surveying the diversity of microorganisms in terrestrial soil environments, which is the main objective of this thesis.

1.2 *Gluconacetobacter xylinus* – a model cellulose producing organism

Cellulose generated from the aerobic, Gram-negative bacterium, *Gluconacetobacter xylinus*, has been used in several recent studies that have utilized the DNA stable-isotope probing technique. The capacity of this organism to generate large volumes of pure cellulose from common laboratory reagents makes it ideal for experiments that rely on isotopically labelled cellulose, due to the fact that many substrates enriched with stable-isotopes such as ^{13}C and ^{15}N are prohibitively expensive. Complex substrates are often unavailable for purchase at all, forcing researchers to harness cell machinery from microorganisms such as this to generate the desired product.

The genus *Gluconacetobacter* produces bacterial cellulose that is chemically identical to the amorphous cellulose that naturally occurs in the cell wall of plant cells (Battad-Bernardo et al., 2004; Kai, 1984). Cellulose produced by bacteria also possesses unique properties such as high mechanical strength, high porosity, enhanced water holding capacity, and high crystallinity

(Czaja et al., 2004). The cellulose fibres produced by *G. xylinus* is free from lignocelluloses and other common components of the woody tissues of plant species, and it is easily recovered in pure form using well-documented techniques (Wiegand & Klemm, 2005). These physical features have made bacterial cellulose an attractive material for academic research and industrial processes (Battad-Bernardo et al., 2004).

G. xylinus is capable of synthesizing extracellular cellulose from various carbon sources including glucose, fructose, arabitol, mannitol, ethanol, glycerol, citric acid, and sucrose (Battad-Bernardo et al., 2004). Acid production is a typical by-product of metabolism of ethanol, D-glucose, and D-xylose in all known strains. Acid formation from sucrose and D-galactose is specific to particular strains (Sievers & Swing, 2005). Production of cellulose can therefore be tracked by measuring changes in pH levels in the culture medium.

Oxygen availability is vital to the capacity of *G. xylinus* to carry out its metabolic functions, and it has been speculated that the film of cellulose produced by these microbes aids them in gaining access to oxygen in the culture medium or natural aquatic environments (Krystynowicz et al., 2002). *G. xylinus* cells are typically found in small clusters, chains, doublets, or as single cells. They possess peritrichous flagella and are highly motile (Sievers & Swing, 2005). It has been postulated that the production of cellulose fibres may also aid the microorganism in generating motion (Koizumi et al., 2008).

1.3 Glycosyl hydrolase enzymes and potential for industry

Glycosyl hydrolases are a broad group of enzymes that are capable of cleaving the glycosidic bond between two or more carbohydrates or between a carbohydrate and another moiety (Coker et al., 2003). As of 2008, greater than 113 different glycosyl hydrolase families have been characterized (Cantarel et al., 2009). The exoglucanases, endoglucanases, and cellobiases (collectively known as cellulases) are all classified as glycosyl hydrolase enzymes. More than a thousand cellulase enzymes have been identified (Duan & Feng, 2010). Cellulases are currently used for a vast array of industrial processes, with demand growing rapidly (Wilson, 2009). By dollar value, it is estimated that enzymes classed as cellulases represent the third largest group of industrial enzymes, accounting for approximately 20% of global industrial enzyme use (Wilson, 2009). Currently, these enzymes are used in the textile, pulp and paper, brewing, and food production industries. Paper recycling, cotton processing, animal feed processing, textile manufacturing, and juice extraction are all examples of industrial processes that utilize these enzymes (Wilson, 2009). The vast majority of industrial enzymes have been obtained from well-studied aerobic cellulolytic fungi, such as *T. reesei* and *Humicola insolens* (Schülein, 1998).

Fermentation of glucose obtained from cellulose fibres has also demonstrated promise for the generation of large volumes of ethanol, butanol, and other hydrocarbons. This has attracted attention, considering the volume of cellulosic materials that are produced as by-products from agriculture and waste products from various human activities. Every year, 150 billion Kg of industrial and domestic waste are generated in the United States alone. Approximately two thirds of this waste is estimated to be biodegradable (Bayer et al., 2007). Increased international energy demand in recent years has stimulated efforts to identify prospective fuel alternatives from renewable sources. Research on converting cellulose waste to fuel has been at the forefront of

these efforts due to the prevalence of waste cellulosic material and the wealth of data that exists in the literature on the basic process. If an economical method for converting cellulose to ethanol or other biofuels can be identified, cellulase enzymes would become the most valuable industrial enzymes in the world (Wilson, 2009). Bioconversion of organic matter to methane via anaerobic fermentation has already been tapped as an energy resource (Krause et al., 2008), with many municipalities employing biogas incinerators at landfill sites. Anaerobic bacteria have also been used to generate hydrogen (H_2) gas from cellulosic biomass (Levin et al., 2006; Liu et al., 2008; Yokoi, 2002).

Although numerous cellulase enzymes have been applied to various industries, substantial demand remains for novel cellulase enzymes that possess unique properties. Much of the research on these enzymes has focussed on the development of thermophilic enzymes that possess high reaction rates (Andrews et al., 2004). However, there are situations where the use of psychrophilic enzymes would be advantageous to industry (Gerday et al., 2000). Gerday et al. (2000) estimated the annual market for thermostable enzymes to have a value of approximately ~$250 million US dollars. Cold-adapted enzymes possess high specific activity, and demonstrate catalytic efficiency that is higher than comparable mesophilic enzymes at temperatures between 0 – 30°C (Gerday et al., 2000). Reaction rates of psychrophilic enzymes can be easily controlled since they denature easily at elevated temperatures (Lee et al., 2006). Enzymes capable of functioning at lower temperatures would also provide cost savings by requiring less energy input to regulate reaction rates (Lee et al., 2006).

Cold-active glycosyl hydrolases would offer significant benefits to the textile and food industries, and could be applied to bioremediation efforts. Treating food products under low

temperature conditions prevents spoilage and affects flavour and nutritional value (Margesin & Schinner, 1994; Nakagawa et al., 2003). Cold-active β-galactosidases have become valuable to the dairy industry in recent years. These enzymes can remove lactose from refrigerated milk at low temperature to allow for milk consumption by people who are lactose intolerant. This is important to the dairy industry, considering that approximately two thirds of the world's adult population may be intolerant to lactose to some degree (Gerday et al., 2000, Nakagawa et al., 2003). Cold-active amylases, cellulases, pectinases, mannanases, and xylanases have also been used to enhance juice extraction, dough fermentation in baking, and as additives for detergents designed for cold washing (Gerday et al., 2000).

1.4 The Arctic tundra – A rich source of cellulosic materials

Soil environments are one of the largest pools of available carbon on the planet (Davidson & Janssens, 2006). The Earth's soils store approximately 1500 gigatons of carbon (Zimov et al., 2006), and the emission of carbon dioxide from soils represents one of the largest fluxes in the global carbon cycle. Soil respiration by microorganisms is the primary pathway by which carbon sequestered by terrestrial vegetation returns to the atmosphere. It has been estimated that respiration by soil microbiota releases 40-68 Pg of carbon per year into the atmosphere (Falkowski, 2000; Lal, 2008). The carbon found in soils is typically in the form of detritus or decaying organic matter (Schlesinger & Andrews, 2000).

High-latitude Arctic soils are unique among soil environments due to the presence of permafrost and their distinct soil structure (Mack et al., 2004). Permafrost is defined as soil that remains at or below 0°C for a period of two years or greater. Permafrost soils are estimated to

cover an area of $22.8x10^6$ km^2, or 24% of the land surface in the northern hemisphere (Zhang et al., 2008). Models have demonstrated that Arctic soil communities possess approximately 13% of the total soil carbon pool, with the majority contained in soil rather than vegetation (Post et al., 1982). Approximately 40% of tundra soil is composed of carbon compounds, principally in the form of the plant tissue polymers lignin and cellulose (Vance & Chapin III, 2001). Much of this carbon is currently inaccessible to decomposing organisms, as it is frozen as part of the permafrost that is characteristic of Arctic soils. The permafrost layer contains plant litter and detritus that has accumulated over thousands of years. It is thought that the upper levels of Arctic permafrost contain upwards of four times the amount of carbon available in the currently active layers (Michaelson et al., 2009). Up to 90% of the total carbon contained within tundra soils is present in the organic horizon and frozen mineral soils (Mack et al., 2004). The fate of this soil organic carbon will have an enormous impact on the fate of carbon within the tundra ecosystem and may have dramatic effects on the global carbon cycle (Zimov et al., 2006).

Recent evidence suggests that tundra soil ecosystems have shifted from a net sink to a net source of atmospheric carbon (Oechel et al., 1993). The input of organic matter from leaf and root detritus has been overtaken by the output of carbon dioxide and methane (Davidson & Janssens 2006). With models indicating that Arctic regions will experience the greatest changes in temperatures as a result of global warming (Schuur et al., 2008), the permafrost melting is a cause for concern. Summer warming in Alaska and Northwest Canada has already accelerated from about 0.15 to 0.17°C per decade (measurements taken from 1961–1990 and 1966–1995) to about 0.3 to 0.4°C per decade (measurements taken from 1961–2004; Chapin et al., 2005; Chapman & Walsh, 1993; Serreze et al., 2000). Warmer temperatures in Arctic regions could lead to the acceleration of organic matter decomposition in tundra soils (Davidson & Janssens

2006). Furthermore, as permafrost thaws, it can lead to lake formation where anaerobic decomposition occurs, generating the greenhouse gas methane (Zimov et al., 2006).

The assertion that warming of the Arctic will inevitably cause further feedback warming of the globe is controversial. Despite extensive research on Arctic soil, a consensus has not been reached regarding the effect of rising temperatures on decomposition. There is debate as to whether the warming temperatures will result in greater decomposition of organic matter, or an increase in fixation by increased vegetation (Davidson & Janssens, 2006; Falkowski, 2000; Melillo et al., 2002). Considering the critical role that microorganisms play in cycling carbon in these systems, understanding the composition and activity of microbial cellulolytic consortia will be integral to the development of models that will help predict future climactic trends.

1.5 DNA stable-isotope probing

It has been estimated that the microorganisms that have been cultured to date represent less than 1% of the actual microbial community in a typical soil sample (Amann et al., 1995; Borneman et al., 1996; Kuypers, 2007), with some estimates reaching lower than 0.1% of all viable cells (Kellenberger, 2001). Considering that most microorganisms have eluded cultivation, inferring *in situ* function from DNA sequences can be challenging for microbial ecologists (Dumont et al., 2006). DNA-SIP is a relatively recent method developed by Stefan Radajewski and colleagues (2000) to help address this problem. DNA-SIP is a particularly useful tool for linking the phylogeny of uncultivated organisms with their function in natural environments (Pinnell et al., 2011). This method allows for the isolation of nucleic acids from organisms that are actively involved in metabolizing isotopically labelled substrates (Dumont et

al., 2006). Typically, these studies rely on the amplification of genes such as 16S and 18S rRNA to generate libraries of organisms capable of using the provided substrate (Dumont et al., 2006).

The first experiments to employ DNA-SIP focused on single carbon compounds such as methane (Radajewski et al., 2000) and methanol (Radajewski et al., 2002). Since then, a number of studies have examined the metabolism of a diverse range of substrates (Table 1.1).

Table 1.1 Summary of major DNA-SIP experiments completed using labelled substrates. When multiple studies have used the substrate, only the first published reference is shown

Substrate	**Habitat**	**Major Phylogenetic Groups Identified**	**Marker Gene Used**	**Reference**
$^{13}CH_4$	Peat Soil	*Methylosinus*, *Methylocystis*, uncultivated methanotrophs from RA-14 group, *Methylobacter*, *Methylomonas*, *Betaproteobacteria*	16S rRNA, *pmoA*, *mmoX*, *mxaF*	(Morris et al., 2002)
$^{13}CH_3OH$	Forest Soil	Uncultivated Alphaproteobacterial methylotrophs, *Acidobacterium*	16S rRNA, *mxaF*	Radajewski et al., 2000
[^{13}C] acetate	Activated Sludge	*Comamonadaceae*, *Rhodocyclaceae*	16S rRNA	(Ginige et al., 2005)
[^{13}C] glucose	Soil	*Arthrobacter*, *Pseudomonas*, *Acinetobacter*, *Massilia*, *Flavobacterium*, *Pedobacter*	16S rRNA	(Padmanabhan et al., 2003)
[^{13}C] cellulose	Soil	*Dyella*, *Mesorhizobium*, *Sphingomonas*, *Myxobacteria*	16S rRNA	el Zahar Haichar et al., 2007
$^{15}N_2$	Soil	*Rhizobiales*, *Actinobacteria*, *Alphaproteobacteria*	16S rRNA	(Buckley et al., 2007)
$^{13}CO_2$	Rice root	*Methanosarcinaceae*, rice cluster-1 Archaea, *Methanobacteriales*	16S rRNA	(Lu & Conrad, 2005)

Adapted from Chen & Murrell, (2011)

Because the DNA-SIP technique is culture-independent, experiments designed to provide data on communities require a substrate enriched with stable-isotope (^{13}C, ^{15}N, ^{18}O). Samples are amended with the substrate of interest and then incubated. Ideally, the amount of substrate provided and the incubation conditions will be as close to *in situ* conditions as possible (Neufeld et al., 2007c). DNA-SIP relies on the incorporation of the stable-isotope into freshly synthesized DNA created by microorganisms undergoing cell division (Neufeld et al., 2007a). Following incubation, isopycnic centrifugation (in cesium chloride) is employed to separate stable isotope-labelled DNA ("heavy" DNA) from unlabeled DNA ("light" DNA). Retrieved heavy DNA can then be used for the characterization and identification of uncultured microorganisms of interest.

1.6 Summary

Most of the natural products used in pharmaceuticals and industry are derived from cultured soil microorganisms (Tomasz & Heald, 2006). The uncultured prokaryotic inhabitants of Arctic soil communities could potentially be storing numerous unidentified natural products of industrial and medical interest. Cellulolysis is an important metabolic process that could potentially be harnessed to generate by-products beneficial to industrial applications (Bhat, 2000). The literature reviewed here demonstrates the potential for recovery of previously unidentified microorganisms involved in the degradation of cellulose in the natural environment. Recent studies (el Zahar Haichar et al. 2007, Bernard et al. 2009) have shown that DNA-SIP is an appropriate method for characterizing and identifying soil microorganisms involved in cellulolysis in soil environments using the 16S rRNA gene as a taxonomic marker.

This thesis describes proof-of-principle research for the DNA-SIP method applied to examine cellulolytic communities in an Arctic tundra soil samples. Arctic tundra was chosen due to its potential to yield previously undiscovered microorganisms and unique cold-weather adapted cellulases of potential commercial value. Understanding the underlying structure and activity of terrestrial microbial communities will also be an important first step towards predicting the effects of changing global climactic patterns (Melillo et al., 2002; Wallenstein et al., 2007). Furthermore, the data presented in this thesis are important as part of an extended collaboration involving metagenomic analysis for enzyme discovery. Once completed, these combined projects will present the first proof-of-principle combination of DNA-SIP and functional metagenomics, in which genes and enzymes are discovered based on function rather than sequence.

Chapter 2: Stable isotope-probing protocol

A modification of this chapter was published in the Journal of Visualized Experiments (Dunford & Neufeld, 2010). An instructional video was also produced as part of the publication process, which is accessible at this website: http://www.jove.com/details.php?id=2027.

DNA stable-isotope probing (DNA-SIP) is a powerful technique for identifying uncultivated microorganisms that assimilate particular carbon and nutrient sources into cellular biomass. As such, this cultivation-independent technique has been an important methodology for assigning metabolic function to the diverse communities inhabiting a wide range of terrestrial and aquatic environments. Following the incubation of an environmental sample with stable-isotope labelled compounds, the extracted nucleic acid must be fractionated to separate nucleic acids of differing densities, followed by the recovery of labelled and unlabelled DNA for subsequent characterization (e.g. fingerprinting, microarrays, gene clone libraries, metagenomics). This chapter describes the process of nucleic acid density gradient ultracentrifugation, fractionation, and recovery of labelled DNA that was optimized for application in this thesis research.

Procedure

2.1 Preparation of reagents

DNA-SIP requires the use of several reagents. Prepare the reagents in advance of the actual procedure. The directions for preparing each reagent are listed in this section.

2.1.1 Cesium chloride (CsCl) solution for preparing SIP gradients – Prepare a 7.163 M CsCl solution by gradually dissolving 603.0 g of CsCl in distilled and deionized water (ddH_2O) to a final volume of 500 mL. Warming the solution slightly while stirring will help dissolve all of the CsCl. Aliquot the final solution in sealed aliquots. In our lab, a common storage practice is to seal 100-mL aliquots in 125-mL serum vials which are then crimp-sealed with butyl rubber stoppers. The sealed aliquots can be stored long term at room temperature (20°C). The seals help prevent evaporation and CsCl "crust" formation. Determine the density of the solution by weighing triplicate 1-mL aliquots, or by using a digital refractometer (e.g. Reichert AR200) that has been carefully calibrated for CsCl solutions. Once calibrated successfully, the Reichert AR200 is consistent and provides accurate readings for several years. At room temperature (20°C), the final density typically ranges from 1.88-1.89 g mL^{-1}, though the density varies slightly each time a new stock is prepared.

2.1.2 Cesium chloride solution for preparing gradients with EtBr - Combine 250 g of CsCl with 250 mL of sterile ddH_2O water. Aliquot this solution to separate serum vials that have been crimp-sealed with butyl rubber seals as described in 1.1

2.1.3 Gradient Buffer – In 800 mL of sterile ddH_2O water, dissolve 121.1 g of Tris base and adjust the pH to 8.0 using concentrated HCl. Adjust final volume to 1 L. Filter sterilize and autoclave this solution and store in smaller aliquots.

2.1.4 Polyethylene glycol (PEG) solution – Prepare the PEG solution by dissolving 150 g of polyethylene glycol 6000 and 46.8 g of NaCl in sterile ddH_2O water to a total volume of 500 mL (30% PEG, 1.6 M NaCl). Autoclave.

Note: The solution separates into two phases with autoclaving. Include a stir bar in the autoclaved bottle so that the solution can be properly mixed when this occurs.

2.1.5 TE Buffer – Using autoclaved stock solutions of 1 M, prepare a solution of 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA (pH 8.0) in sterile ddH_2O water using autoclaved stock solutions of 1 M Tris-HCl (pH 8.0) and 0.5 M EDTA (pH 8.0). Filter sterilize and autoclave.

2.1.6 70% Ethanol – Combine 350 ml of high purity ethanol with 150 ml of sterile ddH_2O water.

2.2 Sample incubation and DNA extraction

For DNA-SIP incubations, samples are typically incubated with heavy-isotope carbon (^{13}C) substrate. Incubation periods and conditions (e.g. nutrient supplementation, moisture, light) will vary depending on the type of sample that is incubated and the nature of the substrate. DNA-

SIP experiments have been successfully performed using single-carbon compounds (e.g. Dumont et al., 2006; Neufeld et al., 2008; Radajewski et al., 2000), multi-carbon compounds (e.g. Bernard et al., 2007, el Zahar Haichar et al., 2007, Padmanabhan et al., 2003), and using labelled nitrogen (e.g. (Addison et al., 2010; Buckley et al., 2007) or oxygen (e.g. Schwartz, 2007). A drawback to using ^{15}N- or ^{18}O-labelled compounds is the decreased separation of labelled nucleic acid, primarily due to the presence of fewer nitrogen and oxygen atoms in DNA and RNA relative to carbon atoms. A critical control for DNA-SIP experiments is an identical incubation established with native (e.g. ^{12}C) substrate. This incubation provides a subsequent comparison to ensure that any apparent labelling of nucleic acid was not an artefact of the ultracentrifugation or G+C content density differences in DNA contributing to separation (Neufeld et al., 2007b). It is also important to keep frozen sample material for comparison to light and heavy DNA, and worth including a no-substrate control to assess background population changes throughout the SIP incubation.

2.2.1 Incubate environmental samples in microcosms containing labelled substrate. The consensus of researchers is that a minimum incorporation of between 5-500 µmol of ^{13}C carbon per gram of sample will be suitable for samples containing high biomass such as soil samples (Neufeld et al., 2007b). For aquatic samples containing less biomass than soils, 1-100 µmol of incorporated ^{13}C carbon per litre may yield a detectable heavy-isotopic signature (Neufeld et al., 2007b). The amount of carbon amendment, proportion of carbon incorporated into biomass and requirement for supplemental nutrient addition for assimilation will all depend on the characteristics of the samples being analyzed and the targeted organisms of interest. A single set of sample incubation guidelines will not be applicable for all samples. Importantly, the substrate concentration used for SIP incubation

should ideally be as close as possible to the concentration normally encountered *in situ*; experimental bias may be a consequence of enrichment culture conditions (Neufeld et al., 2007a).

2.2.2 Following the incubation of sample with stable-isotope labelled substrate, extract DNA from microcosms using a rigorous extraction protocol (for PCR or small insert cloning) or a trusted enzymatic lysis for high-molecular weight cloning (e.g. large-insert metagenomics). RNA co-extraction does not generally affect analysis, so protocols that yield RNA as well as DNA may be used. The ultracentrifugation of the extracted DNA will not shear fragments shorter than ~50 kb pairs (Neufeld et al., 2007b).

2.2.3 Quantify extracted DNA prior to setup of the CsCl gradient ultracentrifugation tubes. Quantify DNA using a spectrophotometer (e.g. Nanodrop® 2000) if the extraction protocol yields only DNA (e.g. column-based kits). Alternatively, quantify using agarose gel electrophoresis.

2.3 Preparing gradient solutions for ultracentrifugation

This procedure involves adding DNA to ultracentrifuge tubes. There are more than one type of tube and rotor so the exact protocol will vary and depending on the manufacturer's instructions. We use a Beckman-Coulter Vti 65.2 rotor with 5.1-ml QuickSeal polyallomer tubes and the protocol will provide the steps and considerations for these conditions.

2.3.1 Using the DNA concentrations determined in step 2.2.3, calculate the required volume of extracted DNA that is required to provide 0.5 µg – 5 µg of DNA in the ultracentrifuge tubes.

2.3.2 Combine extracted DNA (0.5 µg – 5 µg) with Gradient Buffer (see step 2.1.3) and 4.8 ml of 7.163 M CsCl to a total volume of ~6 ml in a sterile disposable 15 ml tube. Note that the density of the CsCl solution can vary even at the same molarity (see step 2.1.1). The following equation can be used to determine the volume of Gradient Buffer/DNA mixture that is required to generate an appropriate missing ratio:

Gradient buffer/DNA volume required = (CsCl stock solution density – desired final density) x volume of CsCl stock solution added x 1.52

Specify the volume of CsCl stock solution at 4.80 ml. The desired final density should be 1.725 g ml^{-1}. The stock solution density was determined in step 2.1.1.

Note also that the relative volumes of CsCl and Gradient Buffer/DNA will result in a combined volume of greater than 5.1 ml. Preparing volumes greater than the maximum volume capacity of the ultracentrifuge tubes (greater than 5.1 ml) will ensure that there is enough solution to completely fill the tube.

2.3.3 Mix by inverting 10 times. DNA is stable at room temperature in CsCl.

2.4. Creating an EtBr control gradient (optional)

Since EtBr is an intercalating dye that complexes with DNA making it visible under UV light, control gradients containing EtBr are beneficial because they provide immediate visual confirmation of gradient formation prior to fractionating of sample tubes. The inclusion of a control tube containing EtBr and a mixture of both ^{12}C-DNA and ^{13}C-DNA (or ^{14}N-DNA and ^{15}N-DNA) allows for immediate visualization of band formation within the tubes upon completion of ultracentrifugation. This is important because a ruptured tube during

ultracentrifugation or improperly programmed run conditions can result in failed gradient formation. Bound to DNA, EtBr changes the density of the DNA and as a result, a different protocol is followed to prepare gradients. Note that other nucleic acid stains can be used instead of EtBr (Martineau et al., 2008) but the protocol will require optimization with other fluorophores.

2.4.1 The control gradient requires two volumes of genomic DNA: one fully labelled with stable-isotope and one without label. We typically use either *Sinorhizobium meliloti* cultured in media containing ^{13}C- or ^{12}C-glucose as the sole carbon source, or *Methylococcus* cultured in the presence of ^{13}C- or ^{12}C-methane as our controls.

2.4.2 Combine a 5-10 µg quantity of both the ^{12}C-DNA and ^{13}C-DNA with Gradient Buffer to a final volume of 1.00 ml in a disposable 15-ml screw-cap tube.

2.4.3 Add 1.00 g of solid CsCl to the same tube. Mix by inversion.

2.4.4 Add 110 µl of a 10 mg ml^{-1} EtBr solution and 4.3 ml of a 1 g:1 mL CsCl stock solution to the same screw-cap tube used in step 2.4.2. The final density of the solution will approximate that of the original CsCl stock solution.

2.4.5 An additional "blank" control solution containing EtBr will also be required to counterbalance the solution created in step 2.4.4. Combine 1.00 ml Gradient Buffer, 1.00 g CsCl powder, 110 µl of a 10 mg ml^{-1} EtBr solution and 4.3 ml of a 1 g ml^{-1} CsCl stock solution in a separate 15-ml screw-cap tube and mix by inversion.

2.5 Ultracentrifugation

2.5.1 Using a bulb and Pasteur pipette, carefully fill ultracentrifuge tubes with gradient solutions prepared in step 2.3.2 (or steps 2.4.2 & 2.4.3 if preparing an EtBr control gradient). Label the tubes on the tube shoulder with a fine permanent marker. CAUTION: Ensure that the tubes are filled exactly to the base of the tube neck. Insufficiently filled tubes are likely to burst during ultracentrifugation.

2.5.2 When all of the required tubes are filled with sample solutions, record the precise mass of each tube. Pair tubes and balance them to within 0-10 mg. For balancing, find nearly matched pairs and add or remove minute quantities of solution until they are balanced, keeping the solution level as close to the base of the tube necks as possible. Note that for weighing tubes, we use an inverted 15-ml screw-cap tube that has been cut in half as a tube holder.

2.5.3 Seal the tubes using a tube "topper" according to the manufacturer's instructions.

2.5.4 Check that the tubes are sealed properly by inverting them and applying moderate pressure. Weigh the tubes again to check that they are still balanced after sealing to within 0-10 mg.

2.5.5 Check each rotor well carefully to ensure that the wells are clean and free of debris or dust that might puncture the tubes during ultracentrifugation.

2.5.6 Insert the tubes into the rotor with the balanced pairs opposite each other. Record the rotor location of each sample – the ultracentrifugation process can cause marker labels to be damaged or erased. Carefully seal the rotor wells as indicated by the manufacturer.

2.5.7 Load the rotor into the ultracentrifuge. Close the ultracentrifuge door and apply a vacuum. If using a Vti 65.2 rotor, set the rotation speed to 44,100 rpm (~177,000g_{av}), the temperature at 20°C, and ultracentrifugation time for 36-40 hours. Select vacuum,

maximum acceleration, and turn off the brake (ensures gradient not disrupted by deceleration). Note that turning off the brake will add an additional 1-2 hours to the run time. Also note that shorter run times may not achieve sufficient band resolution. Long ultracentrifugation runs are recommended, as they lead to greater resolution of the distinct nucleic acid bands.

2.5.8 Immediately upon completion of the ultracentrifugation procedure, remove the rotor carefully. Avoiding any tilting or bumping of the rotor, gently remove tubes from the wells to avoid disturbing the gradients within the tubes. In rare circumstances, a tube will burst during the run. If so, there is a chance that the gradients in the other tubes did not form properly. If a control gradient was included, check this using UV light to confirm gradient formation. If the gradient has not formed properly in the control tube, it is best to repeat all of step 2.5. Note that the EtBr control tube and its blank control may be stored in the dark and reused for up to six months. Take care to clean the rotor carefully according to the manufacturer's instructions once the burst tube has been removed. Do not use damaging metal brushes and cleaners to clean rotor wells! Rotor brushes and cleaner can be purchased from Beckman, the ultracentrifuge manufacturer.

2.6 Gradient fractionation

There are two methods that are currently used to recover DNA from the ultracentrifuge tubes: fractionation and needle extraction. This protocol will only describe the process of extracting DNA using the fractionation technique. This is because for most SIP experiments, labelled DNA cannot be visualized with EtBr and fluorophores and must instead be detected by comparing equivalent "light" and "heavy" fractions from multiple sample tubes. A syringe pump

is highly recommended to retrieve equal density gradient fractions from ultracentrifuge tubes. We use a BSP model infusion pump (Braintree Scientific Inc.). A low-flow peristaltic pump or an HPLC pump may also be used.

2.6.1 Fill a sterile 60-ml syringe with sterile ddH_2O containing sufficient bromophenol blue dye to provide a dark blue colour. Place the syringe on the loading arm of the syringe pump. Attach pump tubing fitted with a 23-gauge 1" needle and turn on the pump until some ddH_2O has come through the end of the needle. Note that any air bubbles in this ddH_2O supply will negatively affect the fractionation process.

2.6.2 Fix one of the ultracentrifugation tubes to a clamp stand. Ensure that the clamp is sufficiently tight to prevent the tube from being displaced but not such that pressure on the tube would cause a release of CsCl solution when the tube is pierced. Pierce the very bottom of the tube along the tube seam using a fresh 23 gauge 1" needle. For the best results, pierce the tube in a controlled, quick, and confident manner. This is very difficult to do well, practice several times before this is first attempted with sample tubes.

2.6.3 For each sample, prepare 12 sterile 1.5-ml microcentrifuge tubes with labels indicating the sample number and fraction (1-12; heavy to light). Using the needle attached to the pump tubing (step 2.6.1), pierce the top of the tube on the upper tube shoulder, along the seam. Collect the gradient solution using the microcentrifuge tubes. As performed for the bottom of the tube, pierce the tube in a quick and controlled manner. Practice beforehand and be very careful to use a controlled pulling motion to prevent the forced needle from passing through the tube and into a finger! Use a previously calibrated pump rate that will yield 12 x 425 µl fractions in 12 minutes (425 µl min^{-1}).

2.6.4 Use a digital refractometer (e.g. Reichert AR200; recommended) or an analytical balance to check the density of fractions from one gradient to confirm proper gradient formation. You will need to use ~50 µl of sample for this test. We often include pure culture DNA in one tube (as described for preparing the EtBr control gradients) to serve as a control for fractionation and use this for density determination. Expect the densities to range from ~1.690 – 1.760 g ml^{-1}, with a median density of ~1.725 g ml^{-1}.

2.7 DNA precipitation

2.7.1 Precipitate DNA from all fractions by adding 20 µg of linear polyacrylamide. Mix by inversion. Add two volumes of PEG solution (see step 2.1.4) and mix by inversion. Note that a carrier for precipitation is critical for quantitative recovery of DNA for gradient fractions but caution should be used if glycogen is used as a carrier for precipitation. Glycogen preparations have been shown to be contaminated with bacterial nucleic acid and contamination can confuse the interpretation of SIP gradient fractions (Bartram et al., 2009).

2.7.2 Leave the tubes at room temperature for 2 hours to allow the DNA to precipitate. If desired, tubes can be left overnight at room temperature.

2.7.3 Centrifuge at 13,000 *g* for 30 minutes. Carefully aspirate and discard the supernatant. A pellet should be visible, but can be very difficult to see at this stage. Work under a bright light source to assist in visualizing the pellet.

2.7.4 Wash the pellet with 500 µl of 70% ethanol. Centrifuge at 13,000 *g* for 10 minutes. Carefully aspirate and discard the supernatant. The pellet will usually be more visible for this step, but will dissociate from the tube wall more easily.

2.7.5 Allow the pellet to dry at room temperature for 15 minutes.

2.7.6 Suspend each pellet in 50 µl of TE buffer (see step 2.1.5). Run 5 µl of each fraction on an agarose gel according to standard lab protocols.

2.8 Fraction characterization

The method used to characterize gradient fractions to assess the success of the SIP incubation will vary depending on the lab and availability of equipment. Targeting a gene such as 16S rRNA, use a method such as terminal restriction fragment length polymorphism (T-RFLP) or denaturing gradient gel electrophoresis (DGGE), to fingerprint all gradient fractions. Using the protocol described above, expect the light DNA to be associated with fractions 9-11 (~1.705-1.720 g ml^{-1}) and the heavy DNA fingerprints to be associated within fractions 5-8 (~1.720-1.735 g ml^{-1}). Unique fingerprints associated with fractions 5-8 of stable-isotope incubated samples, but not with native-substrate incubated controls provides strong evidence linking specific organisms with the metabolism of particular labelled substrate. If insufficient labelled DNA remains for some applications (hybridization, metagenomics), multiple displacement amplification may be used to produce greater quantities (Chen et al., 2010, Binga et al., 2010) but this can introduce chimeras into the amplified DNA (Neufeld et al., 2007b).

2.9 Results

Typical DNA-SIP results will demonstrate a separation of labelled and unlabelled DNA in the gradient formed by ultracentrifugation. Ideally, complete resolution of high molecular weight genetic material (e.g. ^{13}C, ^{15}N) from unlabelled materials will be achieved. Resolution can be witnessed visually by observing band formation in EtBr control tubes. The concentrations

of retrieved genomic DNA contained in the individual gradient fractions may also be used to confirm proper gradient formation.

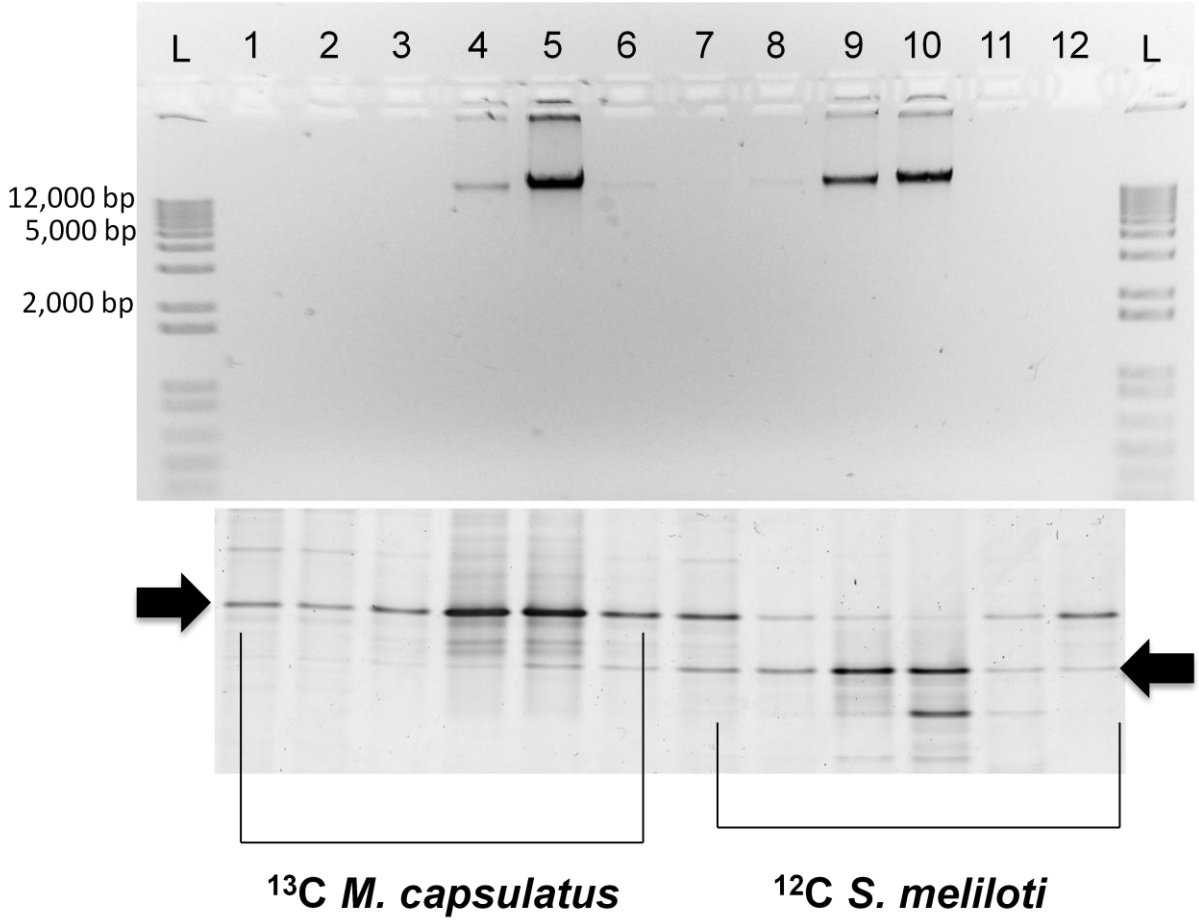

Figure 2.1 Expected gradient fractionation results including two pure cultures. Fraction densities ranged from 1.759 – 1.580 g mL^{-1}, with density decreasing as fraction number increases. Aliquots of gradient fractions were run on a 1% agarose gel from a gradient (top) containing ^{13}C-labelled *Methylococcus capsulatus* and ^{12}C-labelled *Sinorhizobium meliloti*. PCR-amplified DNA from the same fractions was run on a 10% polyacrylamide gel (bottom; denaturing gradient gel electrophoresis, DGGE). Arrows indicate bands corresponding to DNA from the two organisms mixed in this gradient. DGGE fingerprinting reveals distinct pattern differences between fractions 4 and 9.

Figure 2.1 displays representative results of gradient ultracentrifugation performed using two pure cultures. The sample shown in Figure 2.1 was prepared using genomic DNA extracted from *Sinorhizobium meliloti* (Rm1021; Charles et al., 1997), and ^{13}C-labelled *Methylococcus capsulatus* DNA. The labelled and unlabelled genomic DNA associates with specific gradient fractions (Figure 2.1, top). Heavy-isotope labelled DNA can be observed in fractions 4-5, whereas unlabelled DNA is found at high concentrations in fractions 9-10. When shown using the community-based fingerprinting technique denaturing-gradient gel electrophoresis (DGGE; 13), PCR-amplified DNA from the gradient fractions forms discrete banding patterns (Figure 2.1, bottom). Twelve gradient fractions retrieved from the ultracentrifugation tube were loaded on a polyacrylamide gel. The density of the fractions ranged from ~1.580 – 1.759 g mL^{-1}, and they are shown in order of decreasing density. The ^{13}C-labelled *M. capsulatus* DNA formed an intense band in the first six fractions, and was less distinct as the density decreased. The native carbon *S. meliloti* DNA displayed the inverse pattern. The bands containing *S. meliloti* DNA were most distinct in the fractions with the lightest density, and were barely visible in the densest fractions.

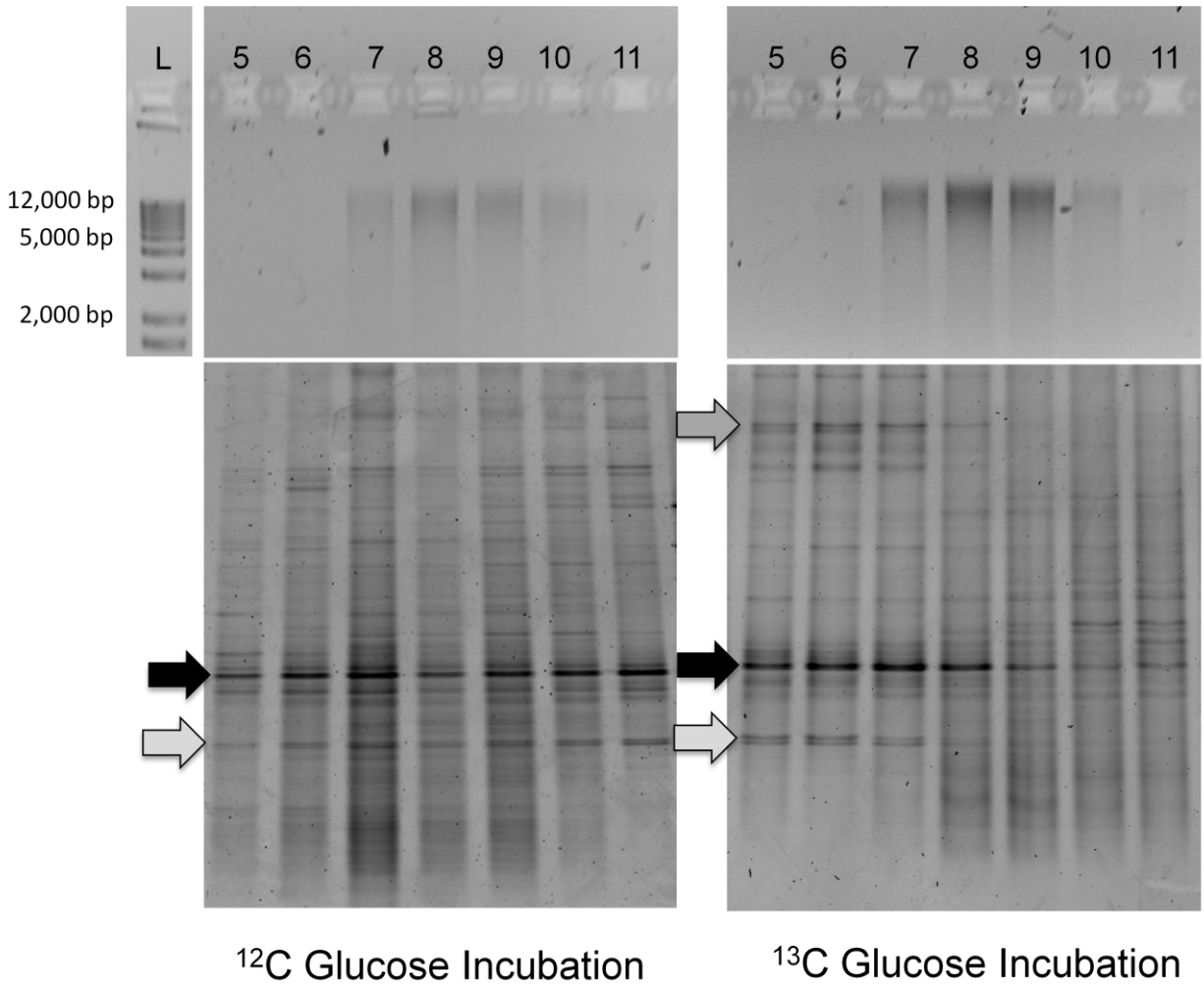

Figure 2.2: Expected results for SIP gradient fractionations from two separate experimental samples. DNA from soil amended with ^{13}C-glucose is shown on the left side of the figure. DNA from soil amended with ^{12}C-glucose is shown on the right side of the figure. Aliquots of gradient fractions from both ^{12}C-glucose amended soil (left) and ^{13}C-glucose amended soil (right) were run on 1% agarose gels. Labelled DNA is not distinctly apparent below the ^{12}C-DNA smear in the ^{12}C-glucose agarose gel. DGGE fingerprints of both communities are shown (bottom). Fingerprinting of fractions reveals enrichment of particular sequences in the ^{13}C-glucose amended sample in fractions 5-8. Arrows represent sequences that are present in the ^{12}C-incubation that are also present in the ^{13}C-incubation in denser fractions. The shift of these sequences to the denser fractions in the ^{13}C-glucose incubation suggests that the source microorganisms actively metabolized the heavy-isotope provided.

Figure 2.2 contains the expected results for SIP gradient fractionations involving environmental samples. Tundra soils from Resolute Bay, Nunavut, were incubated with either

^{12}C- or ^{13}C-labelled glucose for a 14-day period at 15ºC. Figure 2.2 contains data pertaining to the soil sample incubated with ^{12}C-labelled glucose and the soil sample incubated with ^{13}C-labelled glucose. Genomic DNA from the gradient fractions was run on 1% agarose gels for each sample. The methods used to generate the data shown here is described in greater detail in Chapter 3 of this thesis. As shown in Figure 2.2, visible DNA is not distinctly apparent below the ^{12}C-DNA smear in either the ^{12}C or ^{13}C gradient. DGGE fingerprints shown in Figure 2.2 (bottom) reveal completely different patterns between the two samples. This is most readily apparent in fractions 5-8 in the ^{13}C-glucose incubation gel. Of particular interest are the conserved bands indicated by the arrows. These bands are consistent across fractions 5-11 in the sample incubated with ^{12}C-labelled glucose (Figure 2.2). The sample incubated with ^{13}C-labelled glucose demonstrates enrichment for these bands in the denser fractions.

2.10 Discussion

Proper design of stable-isotope probing experiments is of critical importance to achieve clear separation and characterization of labelled DNA; separating labelled sequences from background genetic material is important to maximize the sensitivity of the procedure. Incompletely labelled sequences tend to have reduced resolution in the ultracentrifugation step. The nature of the ultracentrifugation process further compounds issues with reduced resolution. As shown in Figure 2.1, even pure culture nucleic acids have a tendency to distribute across the length of the density gradient. Although *M. capsulatus* is most distinct in the dense fractions of the gel shown in Figure 2.1 (bottom), it is still readily apparent in the lightest fractions. This distribution pattern makes it difficult to accurately identify community patterns, and could result in the inaccurate designation of nucleic acids as light or heavy due to their visible location along

the density gradient. It should be noted that for DNA-SIP incubations involving ^{15}N-labelled substrates, the density difference will be even less (Neufeld et al., 2007b).

Due to the nature of some experiments, isotope incorporation can be poor for a variety of reasons (Neufeld et al., 2007a). Some microbial populations have slow cell-division rates, and thus require long incubation times to reach detectable levels of enrichment. Other populations are capable of metabolizing a variety of substrates, and it is therefore difficult to maximize the incorporation of the labelled substrate. There are also samples that simply yield low levels of total extracted nucleic acid. In cases such as these, the detection of labelled nucleic acids can be difficult.

A variety of natural and synthetic carrier molecules exist that assist in the precipitation and recovery of DNA. Carrier molecules can be biological in origin, such as glycogen (Neufeld et al., 2007b), or synthetic in nature, such as linear polyacrylamide (Applichem). The benefit of using carrier molecules is that they can enable recovery of DNA from CsCl gradients that would not normally be recoverable with high efficiency (Neufeld et al., 2007b). Successful recovery of low nanogram amounts of DNA from CsCl gradients actually requires the use of a carrier molecule (Neufeld et al., 2007b). Recent research has indicated that carrier molecules obtained from biological sources can often be contaminated with DNA from the source organism (Bartram et al., 2009). Therefore it is recommended that synthetic carrier molecules such as linear polyacrylamide be used for DNA-SIP.

Fingerprinting approaches also present their own challenges to assessing enrichment and community diversity of SIP fractions. The amplification of gene sequences in environmental

samples using PCR can induce the formation of chimeras and heteroduplexes (Binga et al., 2008). DGGE gels are only capable of resolving sequences that make up at least 1% of the overall community, possibly obscuring active microorganisms that are rare in the sampled environment. Any contaminating DNA would also interfere with community structure analysis.

If pure cultures are used for DNA-SIP, it is important to know the number of distinct operon copies of the gene of interest the host organism possesses. For example, in Figure 2.1 (bottom), DNA from two pure cultures was used to generate the DGGE profile. Despite the fact that only two organisms are represented in the image, it appears that there are three distinct bands. This is likely a result of the inclusion of *S. meliloti*, as it possesses three copies of the 16S rRNA operon, only two of which are identical (Galibert et al., 2001). This may explain the additional bands visible on the polyacrylamide gel. To confirm that these bands are from the same organism, the bands could be excised from the gel and sequenced.

2.11 Materials

Table 2.1: Specific reagents and materials used in this protocol

Product Name	Company	Catalogue Number	Comments
Reagents			
Bromophenol Blue	Fisher Scientific	BP115-25	
Cesium chloride	Fisher Scientific	BP210-500	
Ethanol, reagent grade	Sigma-Aldrich	652261	
Ethidium bromide	Sigma-Aldrich	E1510	
Hydrochloric acid	Fisher Scientific	351285212	
Linear polyacrylamide	Applichem	A6587	
Polyethylene Glycol 6000	VWR	CAPX1286L-4	
Potassium Chloride	Fisher Scientific	AC42409-0010	
Sodium Chloride	Fisher Scientific	S2711	
Sodium Hydroxide pellets	Fisher Scientific	S3181	
Tris base	Fisher Scientific	BP1521	
Equipment			
Dark Reader	Clare Chemical	DR46B	
Microcentrifuge	Eppendorf	5424 000.410	
Nanodrop® 2000	Fisher Scientific	361013650	
Infusion pump	Braintree Scientific	N/A	Model Number: BSP See www.braintreesci.com for ordering details
Tube sealer	Beckman-Coulter	358312	
Ultracentrifuge	**Beckman-Coulter**		
Ultracentrifuge rotor	Beckman-Coulter	362754	
Ultraviolet light source	UVP Inc.	95-0017-09	Any UV source will suffice
Ultraviolet light face shield	Fisher Scientific	114051C	
Materials			
Butyl rubber stoppers, gray	Sigma-Aldrich	27232	
Centrifuge tubes	Beckman-Coulter	342412	
Hypodermic needle, 23 gauge, 2” length	BD	305145	
Microfuge tubes, 1.5 mL	DiaMed	AD151-N500	
Open center seals, 20 mm diameter.	Sigma-Aldrich	27230-U	
Pasteur pipettes, glass	Fisher Scientific	13-678-6C	
Pipet tips	DiaMed	BPS340-1000	Catalogue number is for 200 µl tips. 10 or 20 µl tips may be purchased from the same source
Pump tubing 1.5 mm bore x 1.5 mm wall	Appleton Woods		
Screw-cap tubes, 15 mL	DiaMed	AD15MLP-S	
Serum vials, 125 mL volume	Sigma-Aldrich	Z114014	
Syringe, 60 mL	BD	309653	

Chapter 3: Characterization of active cellulolytic communities of microorganisms in a tundra soil

3.1 Overview

The consortia of microorganisms responsible for the hydrolysis of cellulose *in situ* are at present poorly characterized. The importance of these communities is underscored by their capacity for converting biomass to greenhouse gases such as carbon dioxide and methane. The metabolic capacities of these organisms is particularly alarming considering the volume of biomass that is projected to re-enter the carbon cycle in Arctic tundra soil environments as a result of a warming climate. Novel cold-adapted cellulase enzymes also present enormous opportunities for a broad range of industries. DNA stable-isotope probing (DNA-SIP) is a powerful tool for linking the phylogenetic identity and function of cellulolytic microorganisms by the incorporation of isotopically-labelled substrate into nucleic acids. By providing ^{13}C-enriched glucose and cellulose to soil microcosms, it was possible to characterize the communities of microorganisms involved in the metabolism of these substrates in an Arctic tundra soil sample from Resolute Bay, Canada. Denaturing gradient gel electrophoresis (DGGE) and 16S rRNA clone libraries were used to visualize changes in community structure and to identify prevalent phylotypes in the different microcosms. Notably, predominant phylotypes changed over time and clustered based on substrate metabolism. Labelled nucleic acids identified by sequenced DGGE bands and 16S rRNA gene clone libraries provided converging evidence indicating the predominance of *Clostridium* and *Sporolactobacillus* in the ^{13}C-glucose microcosms, and *Betaproteobacteria*, *Bacteroidetes*, and *Gammaproteobacteria* in the ^{13}C-cellulose microcosms. Active populations consuming glucose and cellulose were distinct based on principle coordinate analysis of "light"

and “heavy” DNA. A large portion of the recovered sequences possessed no close matches in the BLAST database, reflecting the paucity of data on these communities of microorganisms.

3.2 Introduction

Most life on this planet is dependent on the capture and transfer of light energy to plant biomass via photosynthesis (Rubin, 2008). Cellulose is a major component of the cell wall of plant cells, and is the most abundant organic component of the biosphere (Ross et al., 1991, Lynd et al., 2002, O’Sullivan et al. 2007). Organic matter from plant biomass is a major contributor of carbon to terrestrial soil environments, with global soil carbon content currently estimated at 1500 gigatons (Zimov et al., 2006). The fate of carbon in soil is largely determined by soil microbial communities. Organic matter is mineralized, resulting in the release of CO_2 gas, or incorporated into microbial biomass or humic acids (Bernard et al., 2007). The release of CO_2 from terrestrial soil ecosystems to the atmosphere represents one of the largest fluxes in the global carbon cycle (Falkowski et al. 2000, Lal et al. 2008). Comparative rates of carbon mineralization and carbon storage by soil microflora therefore have a large impact on climatic and atmospheric patterns.

Cellulose hydrolysis is accomplished by three classes of multi-component enzymes: endoglucanases that split β-1,4 glucosidic bonds, exoglucanases that cleave cellobiose or glucose from the non-reducing ends of cellulose fibres, and cellobiases that reduce the resulting cellobiose to glucose monomers (Singh & Hayashi, 1995). Maximal degradation rates have been determined to be the result of three or more cellulase enzymes with different characteristics

acting synergistically. Synergism of extracellular cellulase enzymes from one host (Henrissat et al., 1985) and from multiple organisms (Kato et al., 2005) has been previously demonstrated.

Soil ecosystems retain large pools of carbon (Davidson & Janssens, 2006) and support a diverse range of microorganisms. Soil microbial richness has been estimated at approximately 10 billion microorganisms per gram of soil, representing potentially thousands of different species (Rosselló-Mora & Amann, 2001; Torsvik & Øvreås, 2002). Carbon dioxide emission from soil environments accounts for one of the largest transfers of carbon in the global carbon budget, estimated at 75×10^{15} grams of carbon per year (Schlesinger & Andrews, 2000). Considering that less than 1% of soil microorganisms are readily cultured (Amann et al., 1995), there is also excellent potential for the identification of previously unknown microorganisms and functional enzymes. Cultivation independent approaches have broadened our knowledge of bacterial phylogeny (Riesenfeld et al., 2004), and have yielded novel bacterial enzymes (Duan & Feng, 2010). There is considerable demand for cellulase enzymes that possess novel properties (Bhat, 2000; Wilson, 2009). Enzymes capable of functioning at low temperatures are of particular interest to many industrial sectors, including the textile and food processing industries (Gerday et al., 2000). Several novel cellulase enzymes have already been recovered from environmental samples such as soil, compost, and bioreactors using metagenomics (Duan & Feng, 2010).

Tundra soils environments possess several unique features that make them a likely source of psychrophilic cellulase enzymes. Permafrost soil is characteristic of tundra regions, which is defined as soil that maintains temperatures below 0°C for a period of two years or greater (Zhang et al., 2008). Permafrost layers contain large stores of detritus and litter material from plant biomass, accounting for ~15% of the world's total soil carbon (Post et al., 1982). The abundance

of cellulosic material coupled with the low temperatures associated with polar regions makes tundra soils an ideal ecosystem to search for new and potentially relevant cellulases. Several recent projects have demonstrated the potential for uncovering cellulases with novel properties from polar soils. For example, Coker and colleagues (2003) successfully isolated a β-galactosidase enzyme from an *Arthrobacter* sp. collected in AntArctica. A cellulase was also recently recovered from the culture supernatant of the AntArctic bacterium *Pseudoalteromonas haloplanktis* (Garsoux et al., 2004). Furthermore, several complete genomes of psychrophilic organisms have been constructed (Methé et al., 2005; Médigue et al., 2005), offering opportunities for the discovery and complete characterization of psychrophilic metabolic pathways (Berlemont et al., 2009).

Recent projections indicate that Arctic regions will experience rapid climate changes in the near future (Lawrence & Slater, 2005; Vincent, 2010). As major contributors to biogeochemical cycling and energy flow (Vincent, 2010), Arctic soil microbial communities will have a direct and substantial impact on the fate of carbon currently stored in the permafrost layers. Despite the global significance of microorganisms responsible for the hydrolysis of cellulose in these environments, the organisms involved are poorly characterized. Until very recently, the investigation of cellulolysis in soil has been conducted primarily through cultivation and *in vitro* studies using artificial or modified cellulose substrates (Wirth & Ulrich, 2002). A few recent studies have employed cultivation-independent methods such as DNA-SIP, DGGE, and DNA sequencing in an attempt to provide a more realistic representation of cellulolysis *in situ* (el Zahar Haichar et al. 2007, Bernard et al. 2007, Bernard et al. 2009).

DNA-SIP was pioneered by Radajewski and colleagues (2000) as a technique for identifying microorganisms active in metabolism of particular substrates under conditions that approximate the natural environment. In this method, environmental samples are incubated with stable-isotope labelled substrates of interest. Common stable-isotopes used for these experiments include ^{13}C and ^{15}N (Neufeld et al., 2007b). Organisms involved in the metabolism of the substrate incorporate the isotope into their nucleic acids, making it possible to differentiate active populations from the background organisms. DNA-SIP is reliant on this stable-isotope incorporation; the addition of a fully labelled substrate is critical to the experiment. For my experimental work, cellulose generated by the bacterium *Gluconacetobacter xylinus* was used out of necessity, as ^{13}C-labelled cellulose was difficult to obtain and prohibitively expensive. The DNA-SIP technique was initially used to study microorganisms responsible for the metabolism of one-carbon compounds such as methane and methanol (Radajewski et al. 2000, Morris et al. 2002, Dumont et al. 2006, Neufeld et al. 2007a) and has subsequently been used to study a diverse range of compounds such as acetate (Ginige et al., 2005), glucose (Degelmann et al., 2009; Padmanabhan et al., 2003), carbon dioxide (Lu & Conrad, 2005), and cellulose (el Zahar Haichar et al. 2007, Bernard et al. 2007, Bernard et al. 2009, Lee et al. 2011).

This study represents the first application of DNA-SIP to tundra surface soil environments for characterizing the microbial community responsible for assimilating cellulose carbon. The aim of this proof-of-principle research was to characterize bacterial species involved in both cellulolysis and glucose metabolism in a tundra soil sample from the Canadian Arctic. It was hypothesized that the communities involved in the metabolism of the two substrates (cellulose and glucose) would be different based on the analysis of the heavy DNA, and that the composition of these communities would shift over time as a result of trophic interactions. A

large number of unknown sequences was also expected, due to the relative lack of data on the degradation of cellulose *in situ* and tundra soil communities in general. This report also presents an optimized protocol used to generate maximal amounts of stable-isotope labelled cellulose, and the results of an experiment designed to identify important contributors to cellulolysis in the sample soil. DNA-SIP, DGGE, 16S rRNA gene clone libraries, and principle coordinate analysis (PCoA) were used to characterize populations of microorganisms metabolizing cellulose and glucose over a two-month time period. Fractionated nucleic acids were kept for future experiments related to obtaining enzyme sequences from the communities active in cellulolysis.

3.3 Materials and Methods

3.3.1 Optimization of ^{13}C-cellulose production protocol

The primary objective of this thesis was to generate a proof-of-principle protocol and associated data for characterizing the consortia of microorganisms involved in the hydrolysis of cellulose and related carbon sources in Arctic tundra. In order to assess these consortia, stable-isotope enriched substrates were required to identify organisms of interest. The work presented here was performed in order to determine the best culturing conditions which would lead to maximum recovery of ^{13}C-labeled bacterial cellulose from *G. xylinus*.

3.3.2 Strain cultivation of ^{13}C-labeled cellulose using *Gluconacetobacter xylinus*

Three strains of *G. xylinus* were obtained from collaborating researchers (Table 3.1).

Table: 3.1 *Gluconacetobacter xylinus* test strains

Strain Identity	Source	Strain Characteristic
ATCC 12733	*Dr. W.K. Wan* University of Western Ontario (UWO) 1151 Richmond St., London, ON, Canada, N6A 3K7	Slow growth
KCCM 10100	*Dr. Sang Tae Park* Children's Hospital Boston Harvard Medical School Boston, MA 02115, U.S.A.	Wild type (+)
KCCM 10100 (*mut*)	*Dr. Sang Tae Park* Children's Hospital Boston Harvard Medical School Boston, MA 02115, U.S.A.	Mutant strain defective in GDP-mannosyltransferase

G. xylinus ATCC 12733 was the same strain employed by el Zahar Haichar and colleagues (2007) for their study of agricultural soil communities in Versailles, France. Wild-type (+) *G. xylinus* KCCM 10100 and mutant cells deficient in GDP-mannosyltransferase activity were obtained from Park and colleagues (2006). These mutant cells were created in an attempt to develop a cellulose-overproducing strain of *G. xylinus*.

Bacterial cells were pre-cultured in liquid media containing 2% glucose (w/v). Cells were cultured with native glucose (BioBasic Inc.), or $^{13}C_6$-enriched glucose (Sigma-Aldrich) in liquid media using a modified protocol developed by (Schramm & Hestrin, 1954). The pH of the media was adjusted to 7.0 using hydrogen chloride (HCl). Stock cultures were also maintained on 1.5% agar culture plates containing ^{12}C glucose (Table 3.2).

Table 3.2: *Gluconacetobacter xylinus* growth medium

Reagent Name	Mass Used ($g\ L^{-1}$)	Supplier	Purpose
D-glucose	20	BioBasic Inc. (^{12}C) / Sigma-Aldrich (^{13}C)	Carbon source
Bactopeptone	5	BioBasic Inc.	Nitrogen source
Yeast extract	5	BioBasic Inc.	Nitrogen & amino acid source
Dipotassium phosphate (K_2HPO_4)	2	BioBasic Inc.	Buffer
Agar (plates only)	15	BioBasic Inc.	Solidifying agent

Modified from Schramm and Hestrin, 1954

Bacteria were cultured for approximately 30 days at 30°C under static conditions. Bacterial cellulose was synthesized as an opaque pellicle on the surface of the growth medium. Live cells and contaminating DNA from *G. xylinus* remained in the media after the incubation period. Pellicles were recovered from remaining media for cellulose purification. Each strain was cultured in duplicate 25-mL volumes using the liquid media recipe described in Table 3.2.

3.3.3 Bacterial cellulose purification

A modified version of the cellulose purification protocol described by Schramm & Hestrin (1953) was used to treat recovered cellulose pellicles. Pellicles were removed from media and washed thoroughly using distilled and deionized water (ddH_2O). Washed pellicles were placed in a 1 L Erlenmeyer flask containing a solution of 1% sodium hydroxide (NaOH). The solution was boiled on a hot plate for two hours. The pellicles were then treated with a 5% acetic acid solution for a period of one hour at room temperature. Following treatment with acetic acid, pellicles were washed with ddH_2O for a second time.

DNA contamination of cellulose was assessed by extraction with a FastDNA® Kit (MP Biosciences; Solon, OH, USA) and 16S rRNA gene PCR in addition to direct microscopy. Five µL of genomic DNA extracted from pellicles pre- and post purification was run on a 1% agarose gel stained with 30 µL of GelRed dye for 35 minutes at 85 V.

The PCR used to assess contamination tested DNA extractions with the general bacterial primers 63f (59CAGGCCTAACACATGCAAGTC39, forward) and 518r, (59ATTACCGCGGCTGCTGG39, reverse) and run on an 8% DGGE polyacrylamide gel using a 30-70% denaturing gradient (Green et al., 2010).

Purified pellicles were frozen, and then lyophilized to remove moisture from the cellulose matrix. Following lyophilization, the pellicles were ground into small pieces in a mortar with the addition of liquid nitrogen. The resulting cellulose particles were lyophilized again, in order to remove moisture introduced by condensation formed during liquid nitrogen treatment. The final product consisted of opaque, fibrous cellulose particles of varying size.

3.3.4 Comparison of cellulose pellicle production

Several recent studies (Krystynowicz et al., 2002; Naritomi, 1998) have indicated that the presence of ethanol in the *G. xylinus* growth medium may enhance cell concentrations and overall cellulose production. To assess this claim, the three *G. xylinus* strains were cultured in media containing different nutrient sources. Cultures were grown for seven days in 25 ml of *G. xylinus* media (Table 3.1). In total, six cultures were prepared for each strain, with duplicate cultures assigned to each culturing condition. Basic culture medium (peptone, yeast, dipotassium

phosphate) was amended with either a) 0.5 grams of D-glucose, b) 0.5 grams of D-glucose, 1.75 ml ethanol, 0.25 ml of 5% $MgSO_4$, or c) 0.5 grams of D-glucose, 0.25 ml of 5% $MgSO_4$ solution. Cellulose pellicles were treated using the purification method described above, then air-dried.

3.3.5 Sampling procedures

Arctic tundra soil was collected from Resolute Bay, Nunavut Territory, Canada, on August 17th, 2009. Soil from the organic horizon beneath a vegetated area was collected from the top 10 cm of surface soil. Sieved (4.75 mm) soil was refrigerated and stored for further use. Soil pH, total carbon content, total nitrate content, and soil texture were analyzed by the Soil and Nutrient Laboratory at the University of Guelph (Guelph, Ontario, Canada; see Table 3.4 in Results and Discussion).

3.3.6 Incubation with isotopically labelled glucose and cellulose

Soil microcosms were set up in 100-mL crimp top vials. A total of six incubation vials received ten grams of tundra soil. Two microcosms were used as incubation controls, and therefore no substrate was added to the control vials. The other four vials each received 200 mg of substrate; either glucose or cellulose. The 200 mg mass represented 0.05% of the original soil carbon content. Full details for all microcosms can be found in Table 3.3.

Table 3.3 Experimental soil microcosm contents

Microcosm Identity	Substrate Provided	Carbon Isotope
RB1-AE^{12}CG	Glucose	^{12}C
RB1-AE^{13}CG	Glucose	^{13}C
RB1-AE^{12}CC	Cellulose	^{12}C
RB1-AE^{13}CC	Cellulose	^{13}C
RB1 α Control	-	-
RB1 β Control	-	-

Microcosms were incubated aerobically for a period of 54 days at a temperature of 15°C. Samples of the original tundra soil were kept for molecular analysis. Soil was retrieved from the microcosms on days 14, 28, and 54. On days 14 and 28, two grams of soil was retrieved from each sample vial. On day 54, the remaining mass (~6 g) was recovered from the microcosms. All recovered soil was preserved in a -80°C freezer for long-term storage.

3.3.7 DNA extraction

For DNA extraction, 500 mg from the original soil was mixed with 0.5 mL of CTAB buffer, 0.25 mL of phenol chloroform, and 0.25 mL of chloroform:isoamyl alcohol (24:1) according to previously published protocols (Griffiths et al., 2000). Cells were lysed using a FastPrep® instrument (MP Biosciences Solon, OH, USA) at a speed of 4.0 m s^{-1} for 30 seconds. Precipitation using polyethylene glycol solution (30% PEG 6000, 1.6M NaCl) and ethanol was used to recover nucleic acids from the aqueous phase. Eluted DNA was suspended in 30 µL of sterile ddH_2O. Extracted nucleic acids were visualized and quantified by gel electrophoresis on a 1% agarose gel. All extracted DNA was preserved frozen at – 20°C.

3.3.8 DNA gradient

Ultracentrifuge gradient conditions were optimized using unlabelled *Sinorhizobium meliloti* Rm1021 DNA and ^{13}C-labelled *Methylococcus capsulatus* (Bath). Aliquots of nucleic acids extracted from soil microcosms were centrifuged using the optimized protocol to achieve separation of ^{13}C-labelled DNA. DNA-SIP was carried out as per Dunford and Neufeld (2010; Chapter 2 of this thesis). Samples were centrifuged at a speed of 44,100 rpm (~177,000g_{av}) using a Vti 65.2 rotor (Beckman Coulter, California, USA) at 20°C for 40 hours. Approximately one µg of total genomic DNA was included in each centrifugation tube. Prior to ultracentrifugation, the average density of the prepared gradients was measured using an AR200 digital refractometer (Reichert, New York, USA). Nucleic acids were resolved in a CsCl gradient where the average buoyant density was 1.725 g ml^{-1}.

Samples were fractionated from bottom to top to generate 12 unique gradient fractions. Fractions were generated to retrieve solutions with different buoyant densities, containing light ^{12}C-DNA and heavy ^{13}C-labeled DNA. A BSP model infusion pump (Braintree Scientific Inc, Massachusetts, USA) was used to maintain a constant flow rate. Each of the 12 eluted fractions contained 425 µl of the CsCl/DNA solution. Densities were measured following ultracentrifugation using an AR200 digital refractometer (Reichert, New York, USA). DNA was precipitated from the CsCl gradient solutions using two volumes of polyethylene glycol solution (30% PEG 6000, 1.6M NaCl) and linear polyacrylamide. Precipitated DNA was washed with 70% ethanol solution and suspended in 20 µl of nuclease-free water. Fractions were visualized on 1% (w/v) agarose gels.

3.3.9 Polymerase chain reaction amplification

General bacterial-specific primers 341f-GC clamp (5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGGG CCT ACG GGA GGC AGC AG-3' GC-clamp shown in underlined text) and 518r (5'-ATT ACC GCG GCT GCT GG-3') were used to amplify the V3 region of the 16S rRNA gene sequence (Muyzer et al., 1993). Each PCR amplification was performed with a total volume of 25 µl, which contained 5 pmol of each primer, 0.05 µl of 100 nM dNTP mixture, 15 µg of bovine serum albumin, 0.25 µl of *Taq* DNA polymerase, 2.5 µl of ThermoPol buffer (New England BioLabs, Ontario, Canada), and 1.0 µl of template. Amplification conditions were 95°C for 5 min followed by 30 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min. Final extension occurred at 72°C for 7 min. Generated PCR fragments were ~180 bp in length.

General archaea-specific primers 109f (5'-TTCCGGTTGATCCTGCCGGA-3') and 958r (5'-YCCGGCGTTGAMTCCAATT) were used to amplify archaeal 16S rRNA gene sequences (DeLong, 1992, Jurgens et al., 1997). A nested PCR amplification was performed with a total volume of 25 µl, which contained 5 pmol of each primer, 0.05 µl of 100 nM dNTP mixture, 15 µg of bovine serum albumin, 0.25 µl of *Taq* DNA polymerase, 2.5 µl of ThermoPol buffer (New England BioLabs, Ontario, Canada), and 1.0 µl of template. Amplification conditions were 94°C for 5 min followed by 35 cycles of 95°C for 1 min, 45°C for 1 min, and 72°C for 1 min. Final extension occurred at 72°C for 10 min. A second round of PCR was performed using the primers $SA1f^{GC}$/$SA2f^{GC}$ employed by Nicol et al. (2003) and PARCH519r

(5’-TTACCGCGGCKGCTG) (Øvreas et al., 1997). This amplification was performed with the same settings as those used in the first round of amplification, with the exception of a change in annealing temperature to 53.5°C.

For analysis, 18S fungal gene sequences were amplified using a nested amplification protocol. General eukaryotic primers ITS1f (5’-CTTGGTCATTTAGAGGAAGTAA-3’) (Gardes & Bruns, 1993) and ITS4 (5’-TCCTCCGCTTATTGATATGC-3’) (White et al., 1990) were used for the first round of amplification. Each reaction had a total volume of 25 µl, which contained 5 pmol of each primer, 0.05 µl of 100 nM dNTP mixture, 15 µg of bovine serum albumin, 0.25 µl of *Taq* DNA polymerase, 2.5 µl of ThermoPol buffer (New England BioLabs, Ontario, Canada), and 1.0 µl of template. Amplification conditions were 94°C for 5 min followed by 35 cycles of 94°C for 1 min, 55°C for 50 s, and 72°C for 50 s. Final extension occurred at 72°C for 7 min.

A secondary amplification round was performed using the primers ITS1-F (5’-CGC CCG CCG CGC GCG GGC GGG GCG GGG GCA CGG GGG GCT TGG TCA TTT AGA GGA AGT AA-3’) (Muyzer et al., 1993) and ITS4 (5’-TCCTCCGCTTATTGATATGC-3’) (White et al., 1993). Each reaction had a total volume of 50 µl, which contained 10 pmol of each primer, 0.1 µl of 100 nM dNTP mixture, 30 µg of bovine serum albumin, 0.5 µl of *Taq* DNA polymerase, 5 µl of ThermoPol buffer (New England BioLabs, Ontario, Canada), and 1.0 µl of template. Amplification conditions were 94°C for 5 min followed by 26 cycles of 94°C for 30 s, 57°C for 30 s, and 72°C for 30 s. Final extension occurred at 72°C for 7 min. PCR products were 300-450 bp in size.

All PCR products were analyzed on 1% (w/v) agarose gels stained with ethidium bromide dye (1 mg ml^{-1}). Electrophoresis of agarose gels was performed using a Mini-Sub-Cell GT system (BioRad, California, USA) at 85 V for 35 minutes. Gels were imaged under ultraviolet light using an AlphaImager®HP gel documentation system (Cell Biosciences, California, USA).

3.3.10 Denaturing gradient gel electrophoresis (DGGE)

DGGE was performed on the light and heavy DNA fractions of ^{13}C-incubated microcosms and control microcosms. Samples were recovered at days 14 (2 week), 28 (1 month), and 54 (2 month), and compared to the original soil community. DNA banding profiles from the heavy DNA (Fraction 6; density ~ 1.725 g ml^{-1}) and the light DNA (Fraction 10; ~ 1.708 g ml^{-1}) were used to characterize the communities.

For each sample fraction, 5 µl of PCR product was loaded into one of the lanes of the DGGE polyacrylamide gel. DGGE electrophoresis was performed using the DGGEK-2001-110 system (C.B.S. Scientific Inc., California, USA) according to the manufacturer's directions. Polyacrylamide gels had a denaturing gradient of 30-70% (100% denaturant gradient would consist of 21 g of urea and 20 mL of formamide in a 50 mL total volume) for bacterial products. Archaeal products were run on 10% polyacrylamide gels with denaturing gradients of 15-55%. Following 14 hours of electrophoresis at 85 V, gels were stained with SYBR® Green I nucleic acid gel stain (Invitrogen, Ontario, Canada). Gels were scanned using a Typhoon® 9400 Variable Mode Imager system (GE Healthcare Lifesciences, Quebec, Canada). Specific bands were excised from the gels and re-amplified using the same 341f and 518r primers described, this time

without the associated GC-clamp. PCR products were sequenced using the 518r primer and BigDye terminator version 3.1 (Applied Biosystems, California, USA). Products were run on an ABI 3730xl DNA Analyzer (Applied Biosystems) by the Toronto Centre for Applied Genomics (Hospital for Sick Children, Toronto, Ontario, Canada).

3.3.11 16S rRNA gene library construction

In order to further characterize light and heavy DNA in addition to the original soil community, PCR (with PCR components the same as for DGGE PCR) was conducted with the primers 27f (AGAGTTTGATCMTGGCTCAG) and 1492r (ACCTTGTTACGACTT) (Lane, 1991) as follows: initial denaturation at 95°C for 5 min followed by 30 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min. Final extension occurred at 72°C for 7 min. PCR products from this reaction were cloned into TOPO-TA cloning vectors (Invitrogen) as per the manufacturer's instructions. A total of 96 clones from the original tundra soil library were picked and screened via colony PCR in 50-µl reaction volumes using the -21M13 (TGTAAAACGACGGCCAGT) and M13 reverse (CAGGAAACAGCTATGACC) primers as described by Neufeld et al. (2004). A total of 48 clones from each of the five heavy DNA-SIP fractions and a single light DNA-SIP fraction were collected in a similar manner.

Sanger sequencing of cloned inserts was conducted in one direction with the 27f primer at the Beckman Coulter Genomics sequencing facility in Danvers, Massachusetts, USA. Sequence identities were assigned to all sequences using the RDP Classifier (Wang et al., 2007).

3.3.12 Principle co-ordinate analysis using the QIIME software platform

Sequences were analyzed using the QIIME software package to evaluate relative similarities between the phylogenetic distributions of heavy fractions. Retrieved sequences were clustered using UCLUST (Edgar, 2010) and aligned using the Python Nearest Alignment Space Termination (PyNAST) program (Caporaso et al., 2010). Phylogenetic classification was determined using the RDP Classifier (Wang et al., 2007). Category-based clustering of phylogenetic identities of sequences obtained from the original soil and from the DNA-SIP fractions was performed using principle co-ordinate analysis (PCoA) based on the UniFrac metric. Sequences obtained from the DGGE bands and clone libraries were uploaded into Genbank.

Plot images were generated using QIIME (Caporaso et al., 2010) and are presented using the Kinemage Next Generation (KiNG) molecular modelling program (Chen et al., 2009).

3.4 Results and Discussion

3.4.1 Cellulose production

Stable-isotope labelled substrates can be difficult to obtain, and are often prohibitively expensive. For example, the biotechnology company IsoLife currently offers ^{13}C-labeled cellulose derived from plant sources for approximately $2000 per gram or leaf (IsoLife, Netherlands, http://www.isolife.nl/products.php?letter=), although these products were not even available when this research was initiated. Less complex substrates such as glucose and methane

are typically much more affordable. To compensate for this, researchers who have relied on labelled cellulose (e.g. el Zahar Haichar et al., 2007, Bernard et al., 2009) have used the organism *Gluconacetobacter xylinus* to generate ^{13}C-enriched cellulose from commercially available ^{13}C-glucose.

G. xylinus is a member of the *Alphaproteobacteria* that is capable of synthesizing extracellular cellulose from various carbon sources including glucose, fructose, arabitol, mannitol, ethanol, glycerol, citric acid, and sucrose (Battad-Bernardo et al., 2004). The cellulose that this organism yields is of high purity, and has the added benefit of being free of other biopolymers typically associated with plant-derived cellulose.

Differences were observed between the *G. xylinus* strains that were assessed for cellulose production. Over a one-week incubation period, the KCCM 10100 strains provided a greater mass of cellulose per volume of glucose supplied than the ATCC 12733 strain. More rapid growth was reflected in the greater pH shift of the growth media (Fig. 3.1) and in the overall cellulose yield obtained at the completion of the experiments (Fig. 3.7). Medium pH measurements were recorded every 24 hours for each independent culture. Both KCCM 10100 *G. xylinus* strains demonstrated greater pellicle growth over shorter time periods in comparison to the ATCC 12733 strain. As reported by Krystynowicz et al. (2002), *G. xylinus* typically convert a portion of supplied glucose to keto- and gluconic-acids, resulting in culture media typically reaching a pH of 3.5. Acid production is associated with cellulose production, in *G. xylinus* as it is linked to glucose metabolism in the organism (Sievers & Swing, 2005).

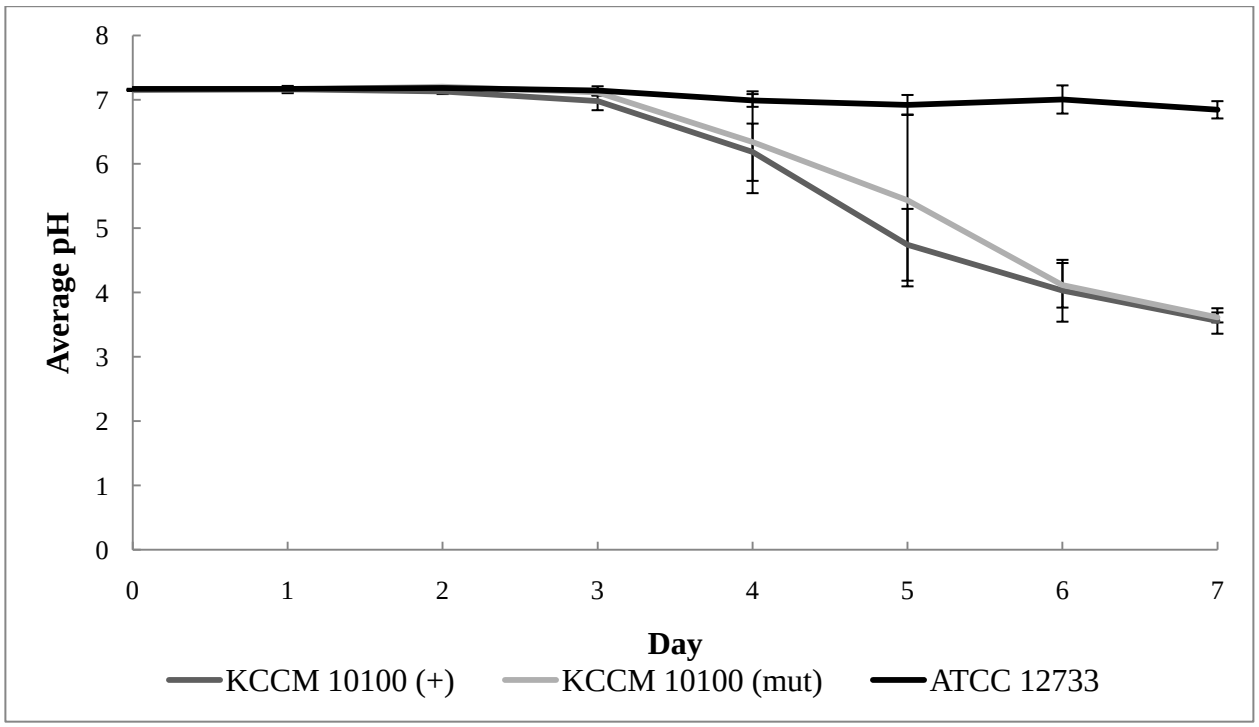

Figure 3.1 Medium acidity during growth of *Gluconacetobacter xylinus* strains over a seven day growth period. Error bars represent the standard deviation of single pH measurements taken from two replicate incubations.

The pH of the medium for both KCCM 10100 strains declined to a pH of 3.5, whereas the pH of the ATCC 12733 strain medium failed to decrease by any appreciable amount. The ATCC 12733 strain was also difficult to culture, due to its slow rate of growth (Figure 3.1). This is reflected in Table 3.4 where no data are shown for this strain in the ethanol and magnesium sulfate trial. The acid production of the KCCM strains was rapid, and the high acidity of the medium helped prevent possible contamination. The mass of cellulose recovered from the KCCM strains from the ethanol comparison trials was greater than the ATCC 12733 strain replicates for all trials (Fig 3.6). Contrary to results reported elsewhere (Park et al., 2006), the mutant KCCM 10100 strain did not seem to provide any enhancement to cellulose production. This strain is deficient in GDP-mannosyltransferase, an enzyme responsible for the biosynthesis

of branched acetan polysaccharides in *G. xylinus*. Park et al. (2006) hypothesized that blocking acetan synthesis would result in overproduction of cellulose.

For the reasons listed above, the wild-type (+) KCCM 10100 strain was selected for ^{13}C-cellulose production in this experiment. Although the highest yields of cellulose were achieved when ethanol and magnesium sulfate were added to the media (as reported by Krystynowicz et al., 2002), I chose to culture *G. xylinus* using only D-glucose and the base medium. Because ethanol contains two carbon atoms, there was a possibility that *G. xylinus* would incorporate this carbon in addition to the stable-isotope carbon found in the glucose. This would have a deleterious effect on the $^{12}C/^{13}C$ ratio of the cellulose produced by *G. xylinus*. DNA-SIP relies on the differentiation of labelled nucleic acids by buoyant density, and thus any dilution of the stable-isotope in the substrate provided would affect the resolving power of the density gradient (Neufeld et al., 2007b). ^{13}C-glucose was added to the base medium and the strains were cultured for a one-month period before cellulose was retrieved.

The modified cellulose washing procedure that was used (Hestrin & Schramm 1953, Krystynowicz et al., 2002) eliminated all detectable nucleic acids from the *G. xylinus* cells. Evidence from direct microscopy, agarose gel electrophoresis, and DGGE polyacrylamide gels all confirmed that the treated cellulose pellicles were free of contaminating DNA (Figures 3.3, 3.4. & 3.5).

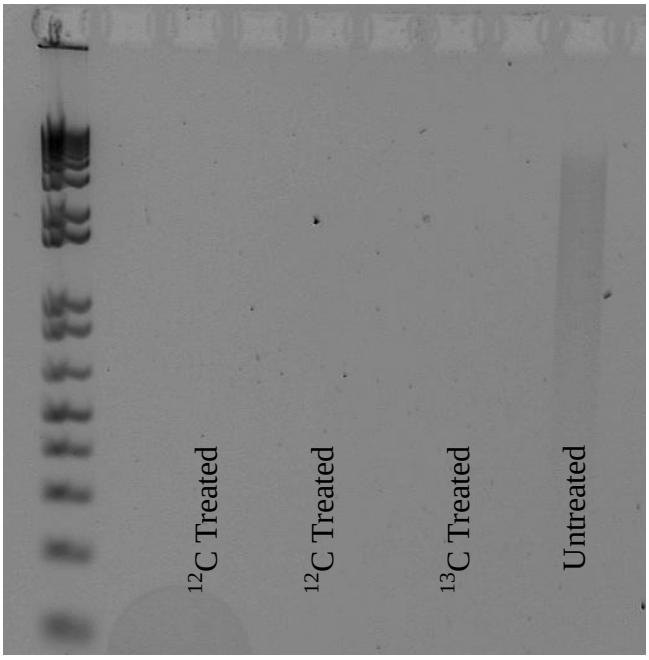

Figure 3.2 Agarose gel electrophoresis image of genomic DNA extracted from treated and untreated cellulose pellicles produced by *G. xylinus.* For reference, 50 ng of 1 Kb PlusTM ladder (Invitrogen, Ontario, Canada) is shown in the leftmost lane.

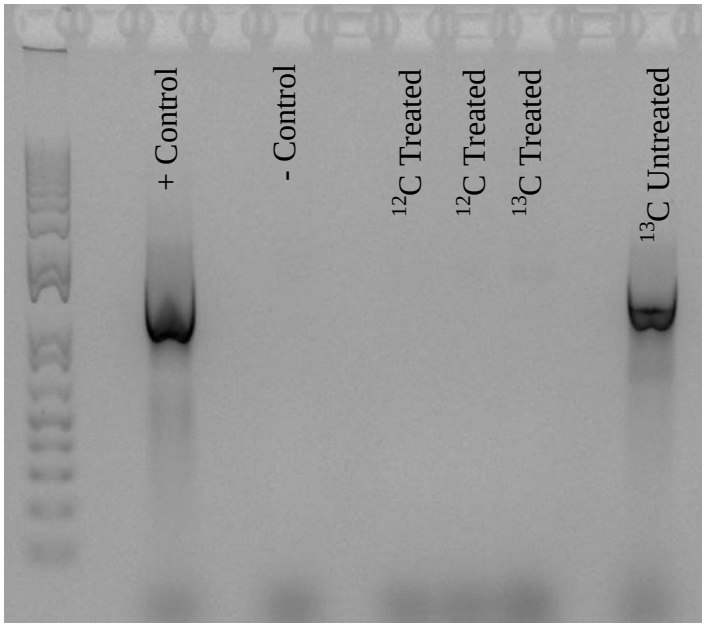

Figure 3.3 Agarose gel electrophoresis image of PCR-amplified DNA extracted from treated and untreated cellulose pellicles produced by *G. xylinus*. The positive control consists of purified DNA from *Methylococcus capsulatus* (Bath). For reference, 50 ng of 1 Kb PlusTM ladder (Invitrogen, Ontario, Canada) is shown in the leftmost lane.

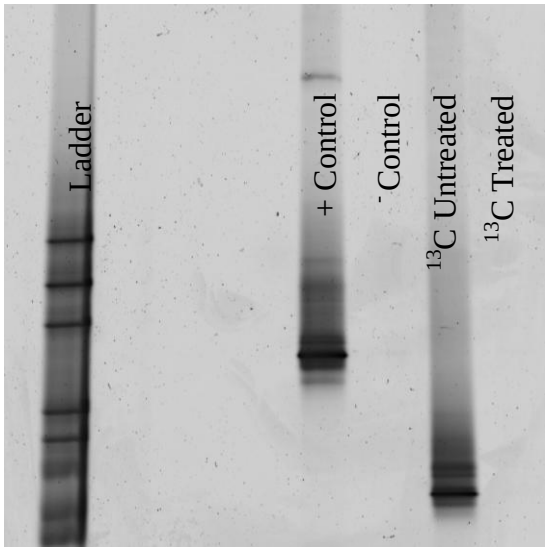

Figure 3.4 DGGE polyacrylamide gel (8%, 30-70% denaturing gradient) showing PCR-amplified 16S rRNA genes from treated and untreated cellulose pellicles. This image was obtained using a Typhoon® 9400 Variable Mode Imager system (GE Healthcare Lifesciences, Quebec, Canada).

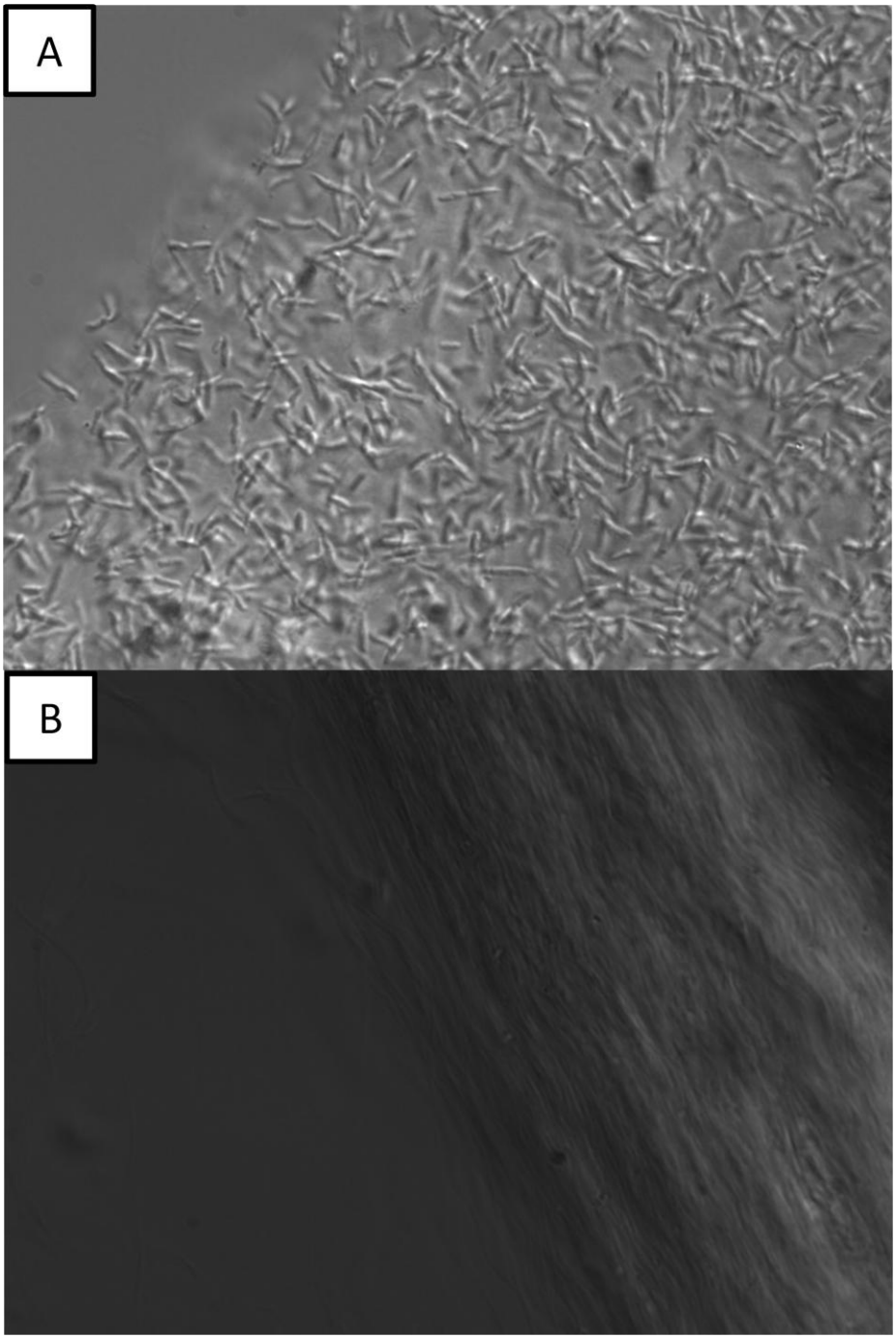

Figure 3.5 Untreated (3.5a) and treated (3.5b) cellulose fibres produced by *G. xylinus* KCCM 10100 magnified at 1000x the original size. This image was obtained using differential contrast microscopy (DIC) using a Zeiss Axiovert 40 CFL microscope (Zeiss Microimaging, NY, USA).

The cellulose pellicles produced and purified were considered to be free of detectable DNA based on PCR, DGGE, and direct microscopy results (Figures 3.4 & 3.5). In Figure 3.5(a), numerous rod-shaped *G. xylinus* cells can be seen. Note the absence of these *G. xylinus* cells in Figure 3.5(b) following the purification method.

3.4.2 Cellulose production by *G. xylinus* during ethanol trials.

Pellicle growth varied between the culturing conditions and between the strains tested. Ethanol appeared to have a positive effect on growth, while the addition of magnesium sulfate alone appeared to have a negligible effect on the mass of cellulose recovered (Figure 3.6)

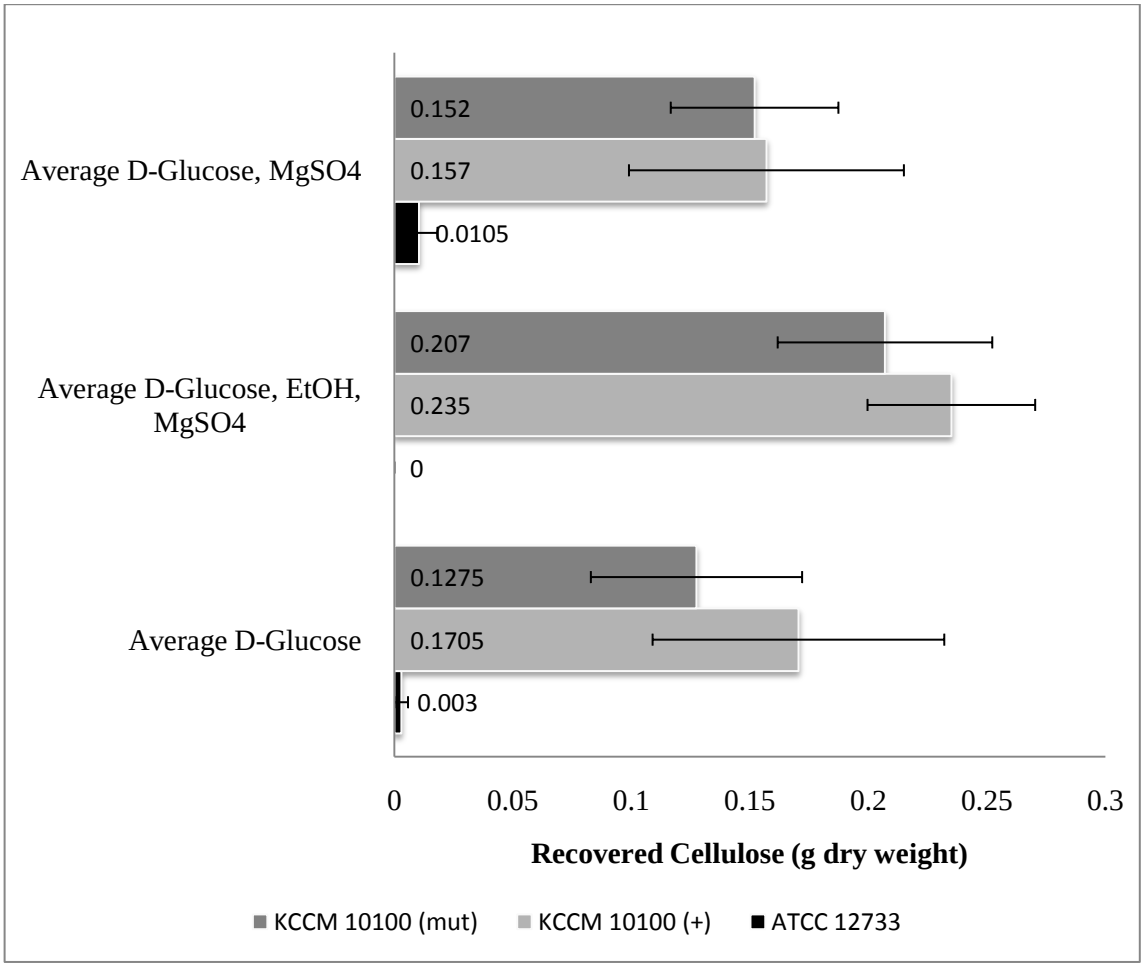

Figure 3.6 Recovered mass of cellulose pellicle post-lyophilization for all strains under each culturing condition. Error bars represent the standard deviation of recovered pellicle mass from two replicate incubations for each culture medium. Pellicles were treated and freeze-dried before weighing.

The KCCM 10100 strains demonstrated superior cellulose pellicle production after a growth period of one week (Table 3.4, Figure 3.6). The ATCC 12733 strain grew slowly and failed to accumulate cellulose. The wild-type (+) KCCM 10100 strain used by Park and colleagues (2006) provided the greatest cellulose yield when cultured in media containing D-glucose, ethanol and magnesium sulfate. Culturing with magnesium sulfate in addition to D-glucose did not have any net effect on pellicle production. The KCCM 10100 (+) strain was chosen for ^{13}C-cellulose production purposes, as it demonstrated the best glucose to cellulose

conversion yield. For ^{13}C-cellulose production, this strain was cultured in media containing only ^{13}C-glucose in addition to the basal medium. Although ethanol addition was found to increase yield, there was concern that unlabelled carbon from the ethanol would be incorporated into cellulose by *G. xylinus* (Battad-Bernardo et al., 2004). Introduction of a ^{12}C-carbon source would potentially reduce the isotopic enrichment of the cellulose and therefore decrease the sensitivity of the DNA-SIP approach.

3.4.3 Microcosm incubations and DNA extraction

The soil used for this experiment was obtained from Resolute Bay, Nunavut, Canada (82°29.7' N, -62°20.1'W). Soil bulk density was calculated to be 0.714 g cm^{-3} (mass of soil/volume). Gravimetric moisture was determined to approximate 82% of maximum water holding capacity based on calculated Water Filled Pore Space (WFPS). This was calculated using the formula shown below:

$$W_S = (M_{AD} - M_{OD})/(M_{OD} - M_T)$$

Where W_S is the water content of air-dry soil, by weight (g g^{-1}), M_{AD} is the mass of air-dried soil and the tray it was weighed in (g), M_{OD} is the mass of oven-dry soil and tin (g), and M_T is the mass of the tray (g) (Ellert et al., 2006). This moisture level was considered sufficient for incubation. The soil nitrate, organic carbon content, pH, and texture are shown in Table 3.5.

Table 3.4 Characteristics of Arctic tundra soil used for incubation.

Test	Result
Soil Nitrate	2.07 mg kg^{-1}
Soil Organic Carbon	43.6 % of dry weight
pH	6.7
Soil Texture (Sand/Silt/Clay; %)	16.6/32.6/50.8

Soil microcosms were incubated at a temperature of 15°C with 200 mg of substrate. This temperature was chosen in order to simulate ambient air temperatures in Canadian high Arctic regions during the summer months. Chapin and colleagues (2005) demonstrate that tundra surface soil reaches temperatures between 20 and 26°C during the summer, further validating this as a reasonable temperature choice for an Arctic tundra surface soil sample. Finally, incubation at this temperature was intended to select for the growth of cold-adapted microorganisms that would theoretically possess enzymes capable of functioning at low temperatures. Psychrophilic enzymes from cold-adapted microbes have excellent potential for use in the detergent and food industries (Gerday et al., 2000).

Previous researchers (Bernard et al., 2009) used a similar mass (50 mg) of labelled substrate to that used during this experiment (200 mg) for their soil DNA-SIP study. Cellulose availability in soil environments is highly transient, and thus there was no defined "natural" level of substrate amendment. Cellulolytic microorganisms are highly variable and tend to be capable of metabolizing a variety of carbohydrate sources in addition to cellulose (Lynd et al., 2002). Thus, 200 mg was considered an appropriate mass of substrate to achieve suitable levels of nucleic acid labelling. To maintain consistency, 200 mg of glucose substrate was supplied, which matched the cellulose amendment. Admittedly, this mass of glucose represents an unnaturally high influx of carbon into a soil system. Hill and colleagues (2008) estimate that the soil glucose

pool undergoes turnover rates of 100-1000 times per day at 20°C. From the same data, the authors estimated that 0.5-4% of soil organic carbon is decomposed to glucose per day, meaning that most of the glucose flux is explained by fluxes between microbial biomass and roots and not as a result of influx of glucose from organic matter (Hill et al., 2008).

From set sampling points, nucleic acids were extracted from microcosm soil according to Griffiths and colleagues (2000). This is a basic bead-beating protocol in which soil is subjected to vigorous shaking with glass beads in order to lyse cells. This is performed in the presence of an aqueous solution (to dissolve nucleic acids) and an organic solution, which denatures and solubilizes proteins. The yield of total DNA extracted from the original soil was estimated at approximately 20 μg g^{-1}. For all incubation dates, extracted nucleic acids were analyzed using agarose gel electrophoresis and DGGE. Total genomic DNA yields from SIP incubations ranged from 1.6-21.0 μg g^{-1}.

Following density gradient ultracentrifugation, the majority of extracted DNA was visualized as a smear between fractions 7 – 11 of the 12 fractions (Figure 3.7). Fractions 6 and 12 were selected to approximate the range of densities containing nucleic acid. These fractions represent a range of buoyant densities from 1.725 g ml^{-1} – 1.700 g ml^{-1}.

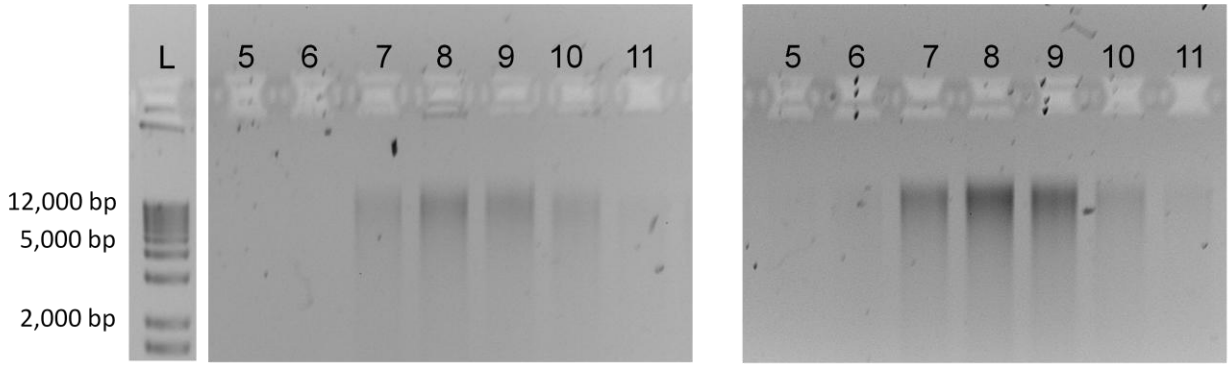

Figure 3.7 Agarose gel electrophoresis images demonstrating the nucleic acid content of DNA-SIP fractions. Fractions are shown in order of decreasing density from left to right, beginning at fraction 5. The 1 Kb Plus ladder (L; Invitrogen, Ontario, Canada) is shown for comparison.

3.4.4 DGGE fingerprinting of fractions

The use of agarose gel electrophoresis was insufficient to properly visualize the small quantity of nucleic acids recovered from the ultracentrifugation tubes in several fractions (see Figure 2.2). Agarose gels were only useful for demonstrating the location of the DNA within the gradient fractions. Denaturing gradient gel electrophoresis (DGGE) was used to assess the general structure of the bacterial community involved in the metabolism of glucose or cellulose in the soil microcosms (Figure 3.8). DGGE gels of the fungal and archaeal community fingerprints did not display any obvious signs of enrichment. Due to the lack of visible enrichment, no further analysis was performed using either the fungal or archaeal primer sets (Figures 3.9 and 3.10).

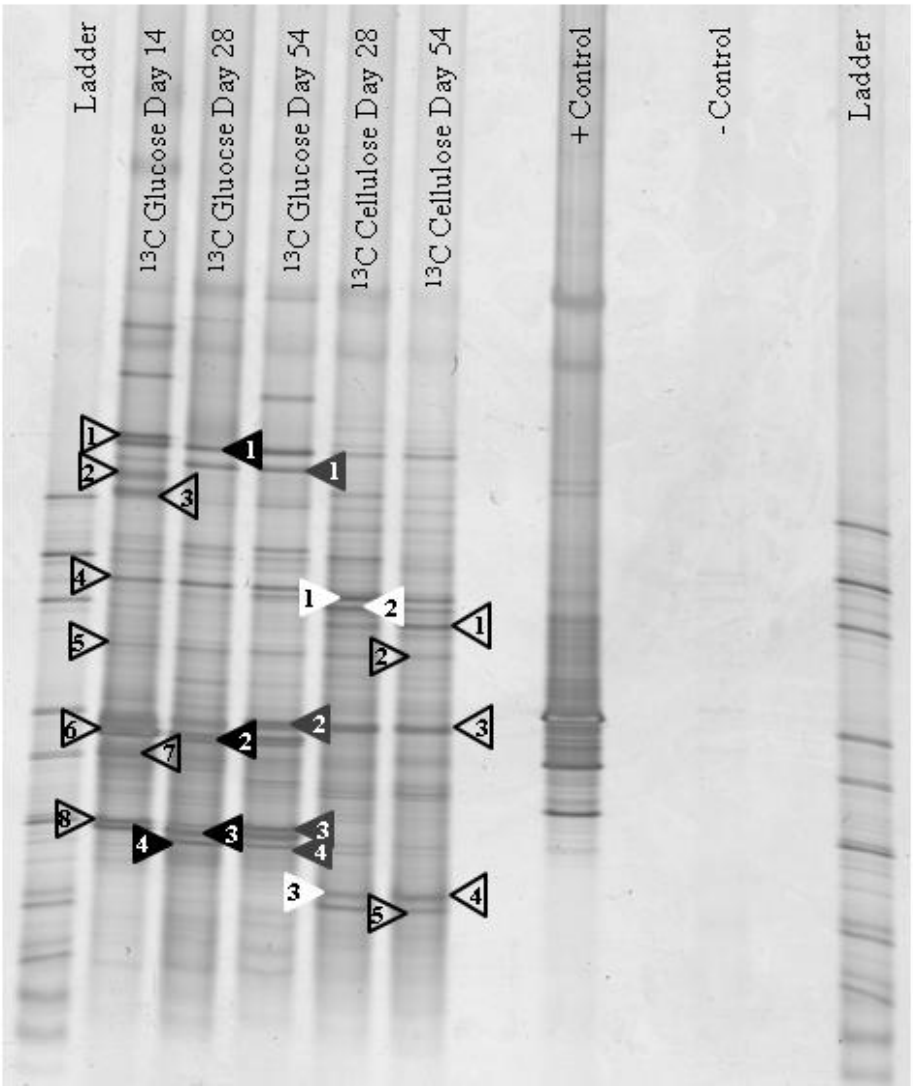

Figure 3.8 A 10% DGGE polyacrylamide gel (30-70% denaturants) containing patterns of soil communities present in extracted DNA fractions generated by the DNA-SIP technique. Fingerprints of communities involved in the metabolism of labelled cellulose and glucose substrate are shown. The numbered arrows indicate community DNA bands that were excised for sequencing. Clear arrows indicate bands excised from the 14-day ^{13}C-glucose incubation (1-8) and bands excised from the 54-day ^{13}C-cellulose incubation (1-5). Black arrows indicate bands excised from the 28-day ^{13}C-glucose incubation. Grey arrows indicate bands excised from the 54-day ^{13}C-glucose incubation, and white arrows indicate bands excised from the 28-day ^{13}C-cellulose incubation. Numbers correspond to the sequences shown in Table 3.6. DNA was retrieved from the heavy fractions of DNA-SIP ultracentrifuge tubes containing nucleic acids extracted from soil microcosms. The source microcosms are labelled in the image. The positive control represents pure culture from *S. meliloti*, and the negative control was run without genomic DNA in order to demonstrate that there was no contamination. DGGE ladders of cloned PCR products are shown in the outermost lanes for comparison.

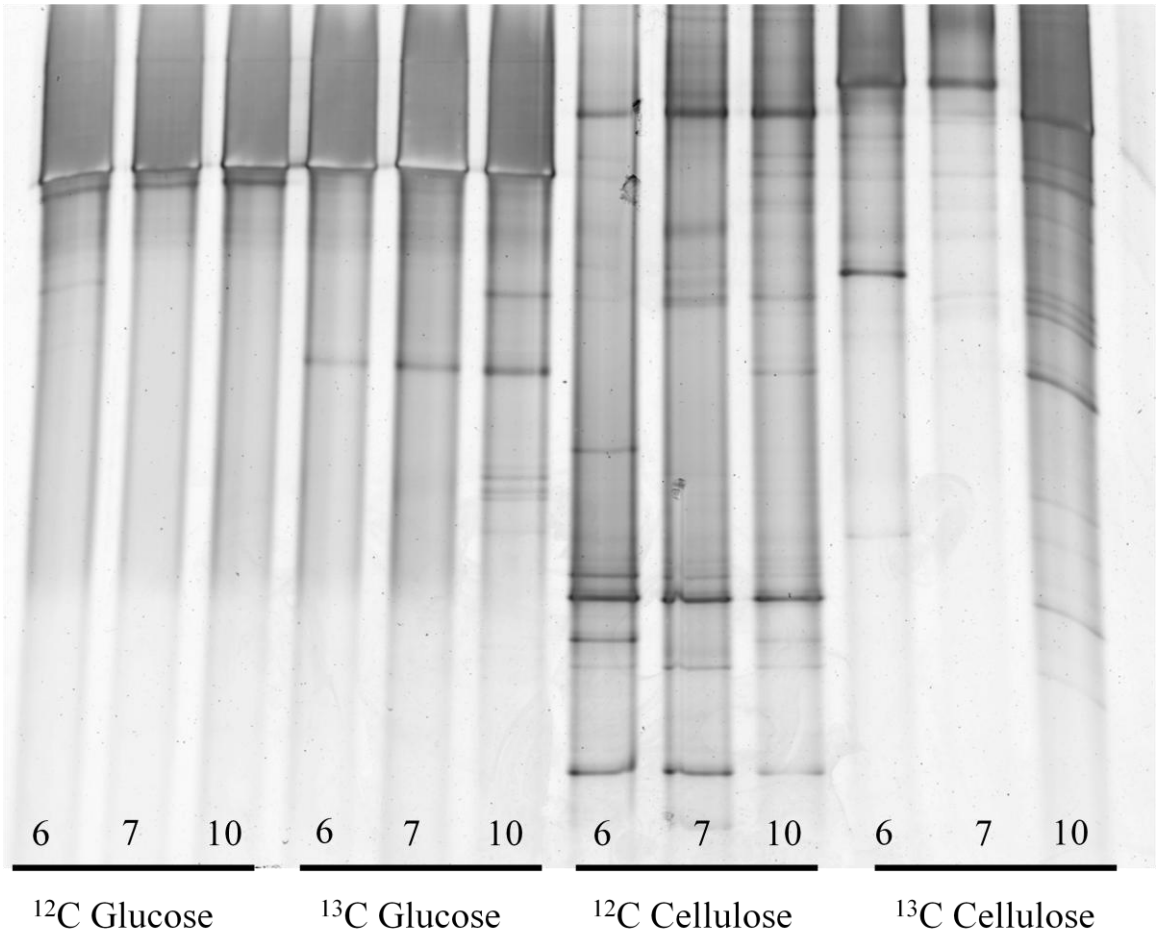

Figure 3.9 A 10% DGGE gel (15/55% denaturants) containing patterns of fungal soil communities retrieved from experimental microcosms after 54 days of incubation. Fractions 6, 7, and 10 were visualized on the gel, representing two dense fractions and one light. Numbers at the base of the figure indicate the fraction that the community DNA was amplified from. Gradient fractions are shown from all microcosms that received substrate.

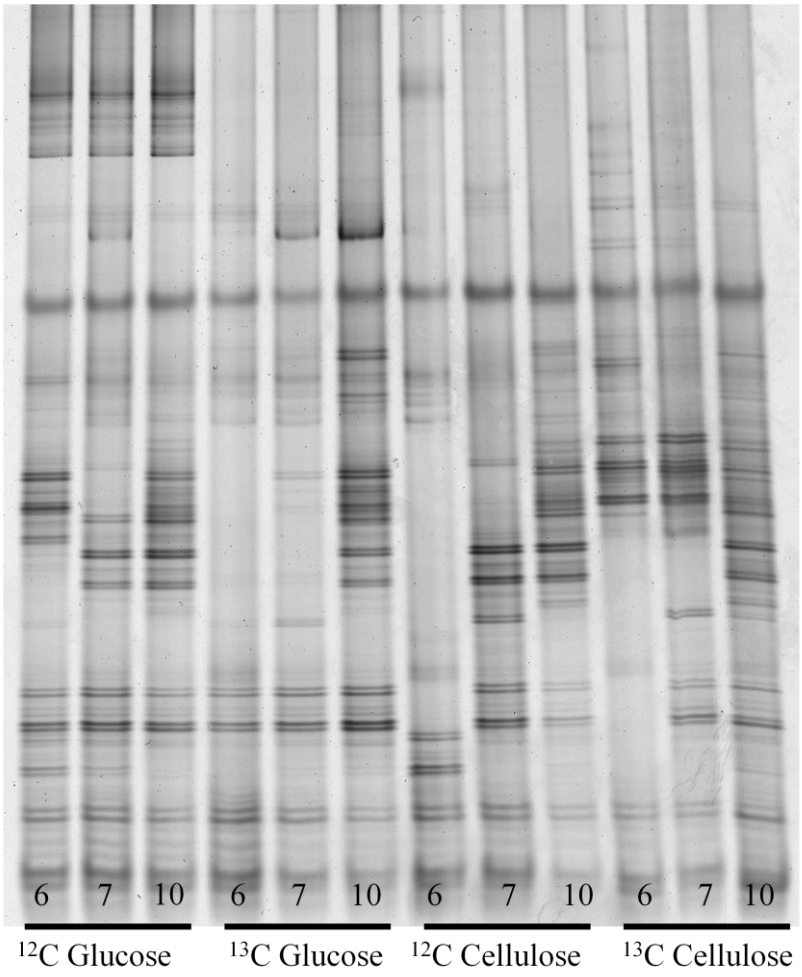

Figure 3.10 A 10% DGGE gel (15/55% denaturants) containing patterns of archaeal soil communities retrieved from experimental microcosms after 28 days of incubation. Fractions 6, 7, and 10 were visualized on the gel, representing two dense fractions and one light. Numbers at the base of the figure indicate the fraction that the community DNA was amplified from. Gradient fractions are shown from all microcosms that received substrate.

The addition of organic matter to the soil microcosms provided a readily available carbon source for microorganisms. For this experiment, cellulose was provided to stimulate the activity of cellulolytic microorganisms. Glucose-amended microcosms were also studied in order to track trophic changes over time. Glucose was a suitable substrate to use, because soluble sugar monomers are the end product of cellulolysis (Zhu et al., 2010). The genetic profile of the active communities was inferred by examining the heavy fractions from the DNA-SIP procedure.

Results indicated that community patterns were highly dependent on substrate amendment. Fingerprints from the heavy fractions of the ^{13}C-glucose incubation were consistent over the incubation period (Figures 3.8 and 3.12). The ^{13}C-cellulose community fingerprints were more variable and demonstrated an increase in sequence diversity in the heavy DNA of the 54-day incubation (Figure 3.13). The increase in diversity may be a result of increased availability of soluble products of cellulolysis such as glucose in the later stages of the incubation period.

Bands were excised from a 10% DGGE polyacrylamide gel (30-70% denaturants) containing PCR-amplified DNA from DNA-SIP fractionations. The community fingerprints shown were retrieved from the heavy DNA present in fraction 6 of the ultracentrifuge tubes. Heavy community DNA samples from the 14-day ^{13}C-glucose microcosm, and the 28-, and 54-day ^{13}C-glucose and ^{13}C-cellulose microcosms were visualized on the gel. Excised bands are indicated by numbered arrows. Retrieved sequences were matched to their closest phylogenetic relatives using the Basic Local Alignment Search Tool, or BLAST (Benson et al., 2000).

Closest relatives of DGGE bands in isopycnically fractioned DNA (^{13}C-cellulose and ^{13}C-glucose treatments)

DGGE band	Length (bp)	Closest relatives		Accession No.	Similarity (Max IDENT) (%)	Alignment	Source
		Microorganisms	Phylogenetic affiliation				
14 Day Glucose 1	134	*uncultured candidate TM7*	*TM7*	HQ595220	100	125/125	Arctic glacier Ice, China
14 Day Glucose 2	135	*uncultured Clostridiales*	*Firmicutes*	GQ355062	97	116/119	Freshwater spring, California
14 Day Glucose 3	141	*Streptococcus mitis*	*Firmicutes*	GU411397	95	136/143	Human oral microbiome, USA
14 Day Glucose 4	148	*Paenibacillae*	*Firmicutes*	FM173788	100	148/149	Tundra core sample
14 Day Glucose 5	134	*Caulobacter*	*α-Proteobacteria*	GU983312	91	88/96	Rhizosphere, NY
14 Day Glucose 6	134	*uncultured Clostridiales*	*Firmicutes*	GQ355062	97	126/127	Freshwater spring, California
14 Day Glucose 7	131	*uncultured Clostridiales*	*Firmicutes*	FM175693	97	121/124	Tundra core sample
14 Day Glucose 8	133	*uncultured Clostridiales*	*Firmicutes*	GQ257691	99	124/125	Groundwater, NJ
28 Day Glucose 1	140	*uncultured candidate TM7*	*TM7*	HQ595220	100	125/125	Arctic glacier Ice, China
28 Day Glucose 2	153	*Sporolactobacillus nakayamae*	*Firmicutes*	HM638427	88	129/146	Wine fermentation, Italy
28 Day Glucose 3	150	*Sporolactobacillus sp. MB-025*	*Firmicutes*	AB548941	94	127/135	Agricultural soil, Japan
28 Day Glucose 4	130	*Clostridia*	*Firmicutes*	AY883101	100	122/122	Molasses wastewater, China
54 Day Glucose 1	153	*Bacillus*	*Firmicutes*	AB243841	98	150/153	Soil/Water, Japan
54 Day Glucose 2	136	*uncultured Clavibacter sp.*	*Actinobacteria*	EF554974	98	133 of 135	Haichar, France
54 Day Glucose 3	144	*Clostridium vincentii*	*Firmicutes*	NR_026336.1	100	119/119	McMurdo Ice Shelf, Antarctica
54 Day Glucose 4	156	*Sporolactobacillus sp. MB-025*	*Firmicutes*	AB548941.1	97	140/144	Agricultural soil, Japan
28 Day Cellulose 1	157	*Paucibacter*	*β-Proteobacteria*	GU213383	98	147 of 149	Deglaciated Alps sand, Slovenia
28 Day Cellulose 2	144	*Cellvibrio*	*γ-Proteobacteria*	AB196321.1	90	118/130	Japanese Soil
28 Day Cellulose 3	150	*Cytophagales*	*Bacteroidetes*	AF141514	100	102/102	Columbia river, WA state
54 Day Cellulose 1	139	*Cellvibrio sp.*	*γ-Proteobacteria*	EU106157	92	131/142	Radish crop, India
54 Day Cellulose 2	135	*uncultured Chloroflexi*	*Chloroflexi*	GQ366602	96	122/126	Himalyan glacier, India
54 Day Cellulose 3	131	*Brevundimonas sp.*	*α-Proteobacteria*	EF486315	95	122/128	Soil, South Korea
54 Day Cellulose 4	148	*uncultured bacterium*	*NA*	HM565375	94	141/149	Mittivakkat glacier, Greenland
	cultured:	*Cytophagales*	*Bacteroidetes*	AF141514	97	99/102	Columbia river, WA state
54 Day Cellulose 5	144	*Cytophagales*	*Bacteroidetes*	AF141514	100	102/102	Columbia river, WA state

Table 3.5 Closest matching GenBank phylogenetic identities of retrieved sequences from DGGE gel excised bands as identified by the BLAST search tool.

3.4.5 Clone library construction

Clone libraries were generated from the original Arctic tundra soil, and from the DNA-SIP fractions of the ^{13}C-glucose and ^{13}C-cellulose soil microcosms. Libraries were constructed from the heavy DNA from fraction 6 of the ^{13}C-glucose microcosm taken on days 14, 28, and 54, and the DNA from fraction 6 of the ^{13}C-cellulose microcosm taken on days 28 and 54. A library was also constructed from fraction 10 (light DNA) of the ^{13}C-cellulose microcosm collected on day 54. This library was intended to serve as an internal control. The nucleic acids in this fraction should theoretically be unlabelled, and therefore should approximate the background community of bacteria that were not involved in substrate metabolism. In total, 396 clones were generated, of which 374 sequenced successfully. Figure 3.11 shows the relative distributions of phyla within each of the seven clone libraries.

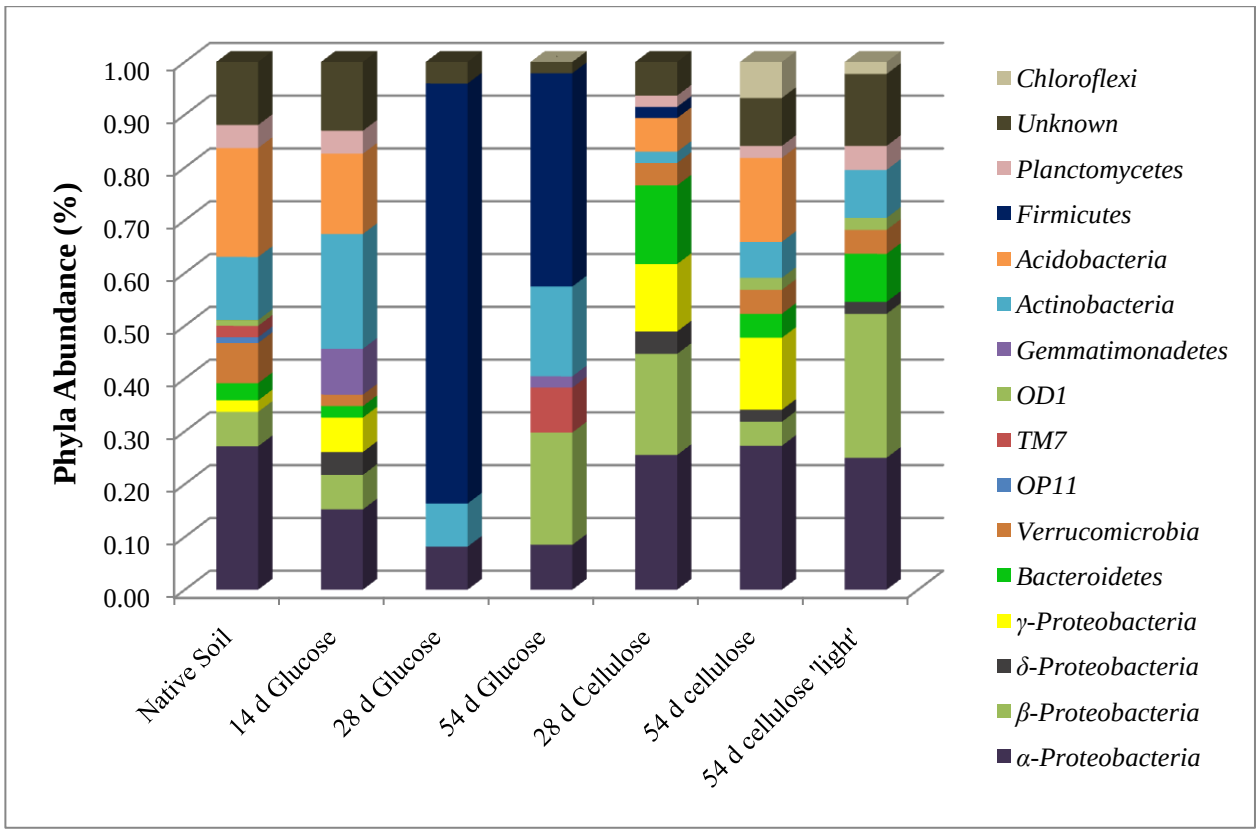

Figure 3.11 Relative distributions of bacterial phyla within each clone library.

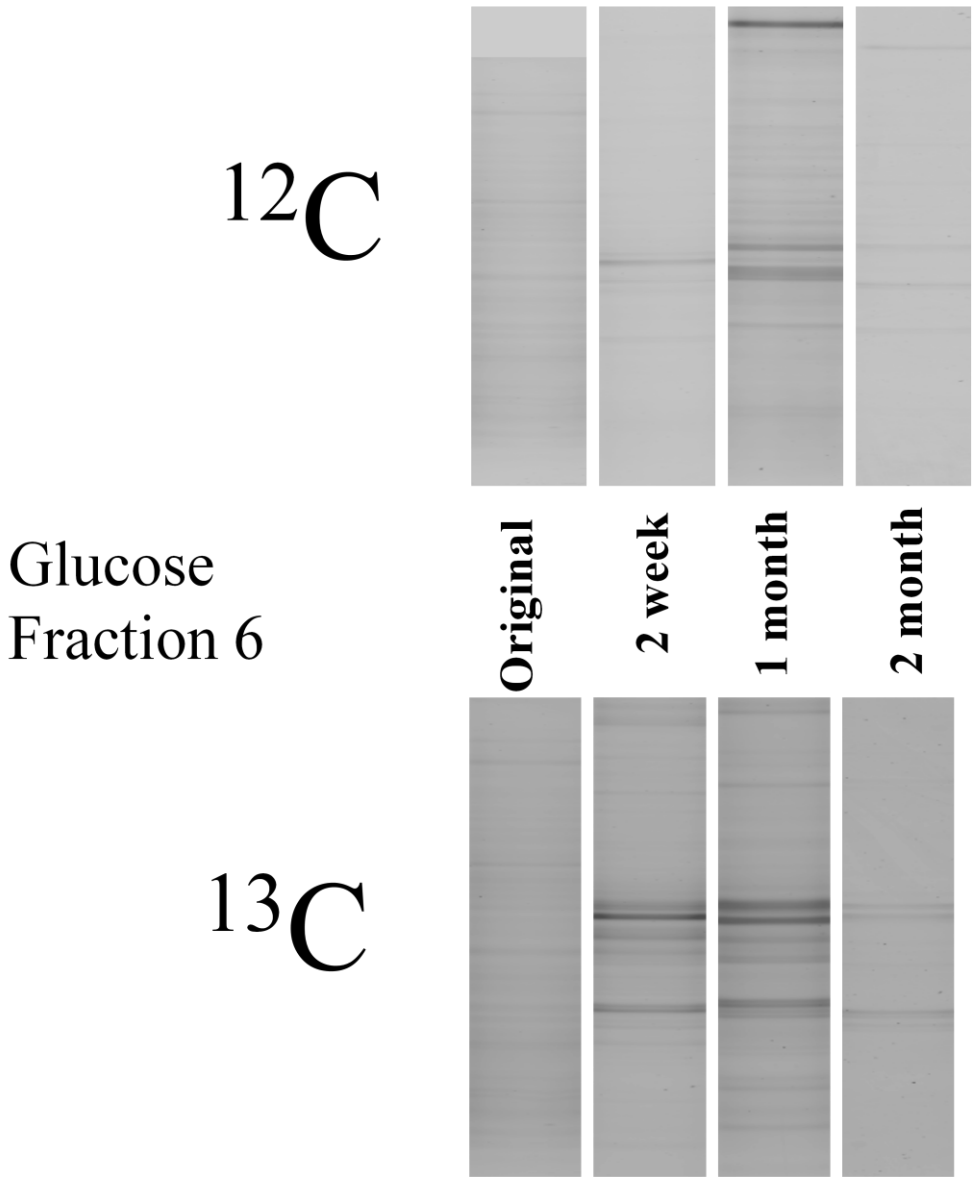

Figure 3.12 Changes in community composition of the heavy and light fractions of DNA of the ^{13}C-glucose microcosm over time as revealed by the DGGE fingerprinting technique

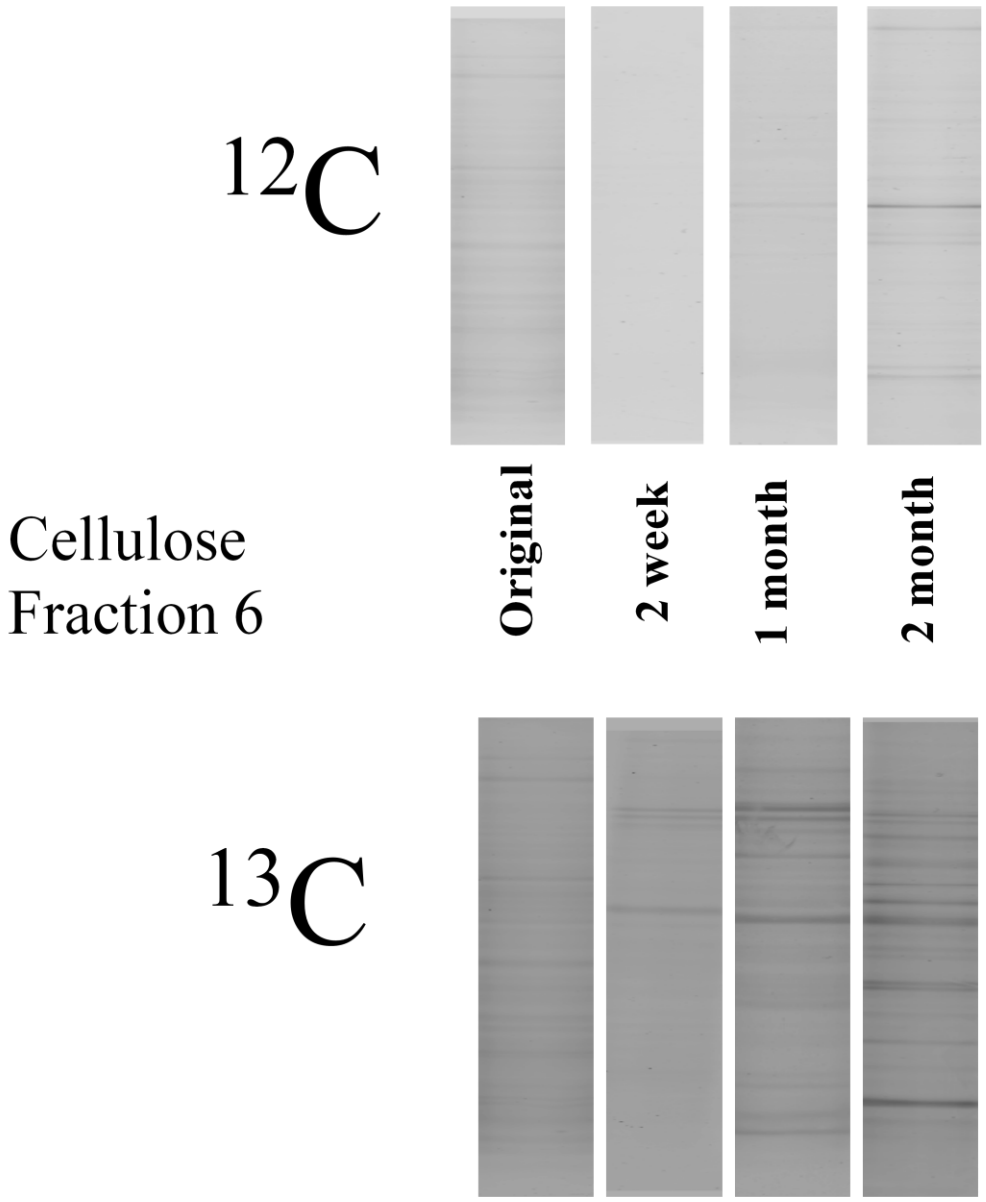

Figure 3.13 Changes in community composition of the heavy and light fractions of DNA of the ^{13}C-cellulose microcosm over time as revealed by the DGGE fingerprinting technique

As shown in Figures 3.12 and 3.13, the ^{12}C incubations provide a baseline for the comparison of the communities. The composition of the heavy DNA in fraction 6 changes, despite the fact that no stable-isotope labelled substrate was provided. The DGGE bands from these incubations do however show less diversity in comparison to the incubations that were amended with ^{13}C substrate. Fraction 6 from the ^{13}C glucose incubation demonstrates rapid

growth of particular phylotypes including *Sporolactobacillus* and *Clostridium* which are not noticeably present in the ^{12}C incubations. A similar trend is observed in the cellulose incubations, as the ^{13}C-cellulose microcosm band profiles differ from the ^{12}C-cellulose control. This is particularly evident in the 54-day incubation, where the ^{13}C-cellulose fingerprint demonstrates a much broader diversity than that of the ^{12}C-cellulose microcosm. The change in the communities over time demonstrates the metabolism of the substrates provided. The ^{13}C-glucose was metabolized rapidly, based on the rapid change in the DGGE fingerprint taken from fraction 6 of the ^{13}C-glucose amended microcosm. Enrichment of the cellulolytic community appeared to have progressed more slowly, possibly as a consequence of the relative recalcitrance of the substrate to degradation.

Sequence libraries demonstrating increased abundance of *Firmicutes* bacteria from the heavy DNA fractions taken from the days 28 and 54 ^{13}C-glucose incubations. The large contribution of *Firmicutes* sequences in the 28d and 54d glucose libraries in Figure 3.11 is somewhat misleading. As shown in Table 3.6, the primary contributors to the *Firmicutes* phyla in the ^{13}C-glucose microcosm collected on day 28 were *Sporolactobacillus* sequences, while the primary *Firmicutes* sequences isolated from the ^{13}C-glucose microcosm on day 54 were associated with *Clostridium*. Sequences from the heavy DNA ^{13}C-cellulose fractions retrieved on days 28 and 54 demonstrate high abundance of *Bacteroidetes*, *Gammaproteobacteria* and *Betaproteobacteria* (Figure 3.11).

The composition of sequence libraries was very similar to what was observed in the analysis of the DGGE band identities. Excised bands from the glucose incubations demonstrate the prevalence of *Sporolactobacillus* and *Clostridium* in heavy DNA associated with ^{13}C-glucose

incubations (Table 3.5). The 16S rRNA gene libraries for the glucose microcosms show a high abundance of these taxa in the 28-day and 54-day libraries. Similarly, the band sequence identities from the ^{13}C-cellulose heavy DNA communities are reflected in the community composition changes represented in the clone libraries. Sequences closely affiliated with the *Bacteroidetes*, *Gammaproteobacteria*, and *Betaproteobacteria* were identified using BLAST, and these taxa were also shown to be more prevalent in the 28-day and 54-day ^{13}C-cellulose heavy DNA fractions. These results provide converging evidence demonstrating the assimilation of carbon derived from isotopically labelled substrates into active members of the tundra soil communities.

The shifts in community diversity also suggest trophic interactions and succession patterns as a result of substrate metabolism. This is supported through comparison to unfractionated bulk soil DNA from the original sample (Figures 3.12 & 3.13). The enriched samples display distinct differences in comparison to the original population. The inclusion of a clone library from a fraction containing unlabelled DNA also supports the success of the DNA-SIP technique. Excised DGGE bands and the clone libraries both revealed differences in community structure as a result of substrate amendment. The UniFrac metric was used to assess the effectiveness of the DNA-SIP technique in differentiating active microorganisms from the background bacterial community.

3.4.6 UniFrac analysis using the QIIME software platform

The unique fraction metric (UniFrac) measures sample-specific phylogenetic distance between taxonomic groups in a phylogenetic tree (Lozupone & Knight, 2005). Essentially, this

metric identifies the amount of evolutionary adaptation unique to each sample by representing the evolutionary divergence between the communities. Similar communities would demonstrate much of the community possessing similar phylogenetic representation, whereas in divergent communities, distinct lineages would be more apparent in both communities (Lozupone & Knight, 2005).

Combined with PCoA, the UniFrac metric is useful for the present study, as it provides a method for determining if the phylogenetic lineages present in the SIP heavy DNA samples were distinct from one another (Lozupone et al., 2010). This information can be used to infer factors responsible for the apparent differences between microbial communities and demonstrate the phylogenetic groups that differentiate samples. In this case, the underlying hypothesis is that substrate amendment will fundamentally alter the phylogenetic diversity of the soil microcosms by selecting for microorganisms capable of metabolizing the substrate provided.

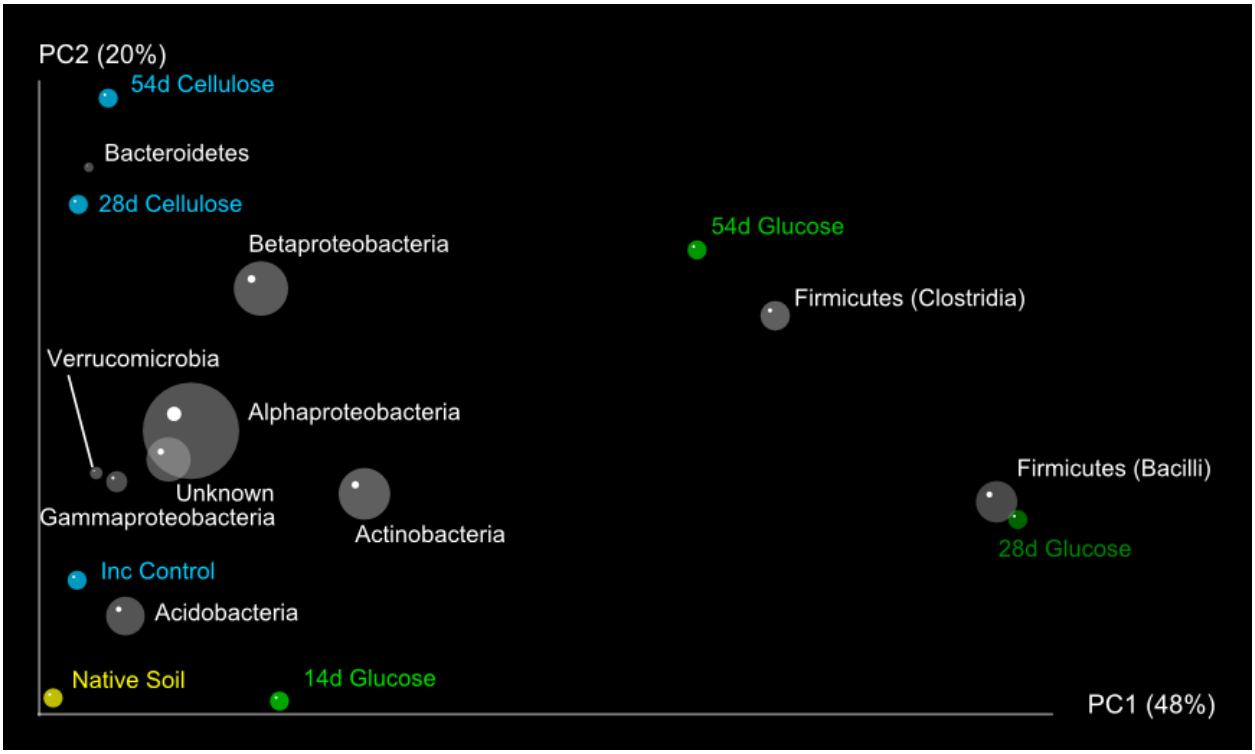

Figure 3.14 PCoA clustering of 374 bacterial clone sequences retrieved from soil SIP incubations using an unweighted UniFrac analysis. The original soil library is shown in yellow. The cellulose-amended libraries are shown in light blue, while the glucose-amended libraries are shown in green. The incubation control ("Inc Control") designates the library that was constructed from the light DNA of the 54-day cellulose incubation. Sequences were analyzed at the class level. For most taxa, it was possible to classify the sequences at the level. Labels for these lineages are given at the taxonomic level where a confident classification could me made using the RDP Classifier (Wang et al., 2007). This biplot also shows grey spheres that demonstrate the top ten bacterial classes responsible for sample differentiation. One of the classes is labelled 'Unknown', indicating that taxonomic identification at the class level was impossible for these sequences using the classifier. The size of the spheres reflects the abundance of that group in the sequence data. The location reflects the importance of that group in differentiating samples.

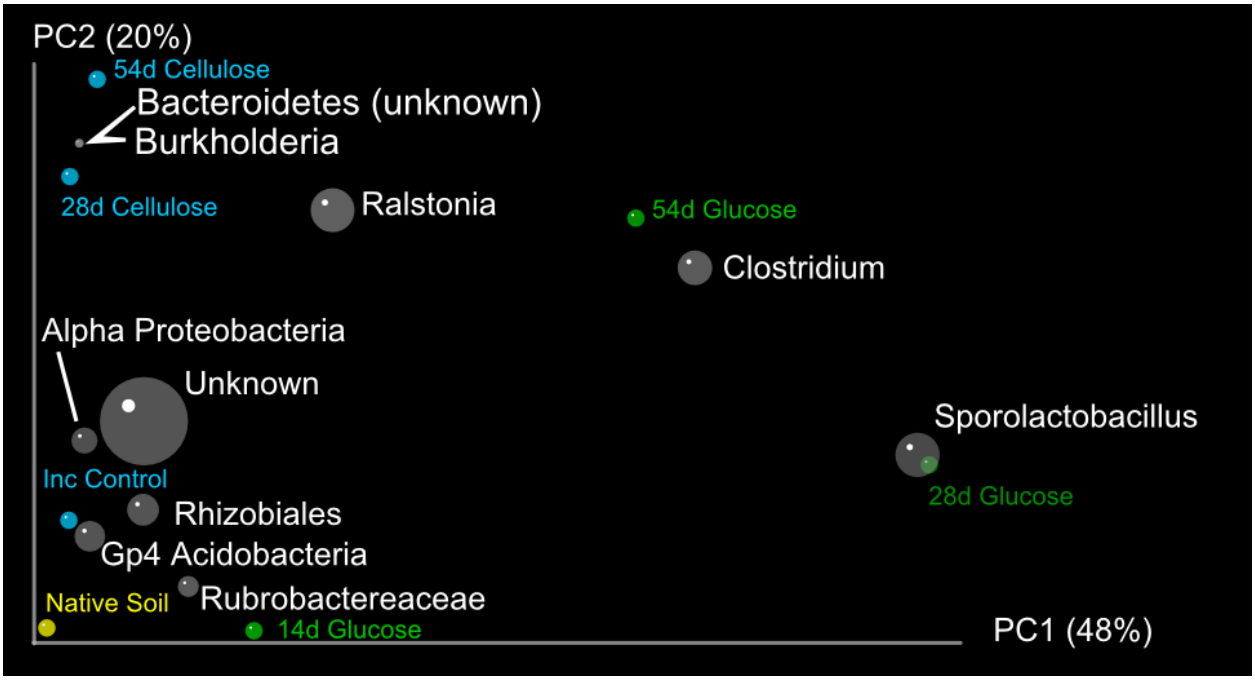

Figure 3.15 PCoA clustering of 374 bacterial clone sequence identities retrieved from experimental soil microcosms using unweighted UniFrac analysis. The original soil library is shown in yellow. The cellulose-amended libraries are shown in light blue, while the glucose-amended libraries are shown in green. The incubation control ("Inc Control") designates the library that was constructed from the light DNA of the 54-day cellulose incubation. This biplot also shows grey spheres that demonstrate the top ten bacterial taxa responsible for sample differentiation. Sequences were analyzed at the genera level. For most taxa, it was impossible to classify the sequences to the genera level. Labels for these lineages are given at the taxonomic level where a confident classification could me made using the RDP Classifier (Wang et al., 2007). The size of the spheres reflects the abundance of that group in the sequence data. The location reflects the importance of that group in differentiating samples.

Table 3.6: The top fifteen phylogenetic lineages that were identified as evolutionarily unique to one of the soil microcosms by the UniFrac metric. These lineages were used as the basis for clustering using the unweighted UniFrac metric. Sequence abundance in each microcosm is also shown.

	Sequence Prevalence in Microcosms						
Lineage	Native Soil	14 day Glucose Heavy	28 day Glucose Heavy	54 day Glucose Heavy	28 day Cellulose Heavy	54 day Cellulose Heavy	54 day Cellulose Light
Sporolactobacillus	0	0	24	1	0	0	0
Ralstonia	0	1	0	10	1	8	0
Clostridium	0	0	2	8	0	0	0
Gp4 *Acidobacteria*	4	0	0	0	1	0	3
Burkholderiales incerta sedis 5	0	0	0	0	5	3	0
Caulobacter	0	1	1	1	1	2	0
Bacteroidetes	0	0	0	0	4	2	0
Clostridium	0	0	1	4	0	0	0
Unknown	0	4	0	0	0	0	0
Verrucomicrobia Subdivision 3 genera incertae sedis	1	0	0	0	0	0	0
Hyphomicrobiaceae	1	1	0	0	1	0	0
Cellvibrio	0	0	0	0	3	0	0
Alpha-proteobacteria	0	0	0	0	3	0	0
Rhizobiales	3	0	0	0	0	0	0
Gp4 *Acidobacteria*	0	2	0	0	1	0	0

Two distinct community clusters were identified based on metabolic activity, at both the class and genera levels (Figures 3.14 and 3.15 respectively). Sequences from cellulose incubations retrieved after 28 and 54 days cluster with each other (blue), as do sequences retrieved from the 28- and 54-day glucose incubations (green). Sequences obtained from the 14 day glucose incubation and the incubation control (light DNA from the 54 day cellulose incubation) were similar to the original soil library (yellow). At the class level, these clusters

corresponded to microcosms with either an increased abundance of *Firmicutes*-type organisms or increased abundance of *Bacteroidetes* and *Betaproteobacteria*. The differentiation of the clusters based on these classes of organisms is supported by the increase in their abundance in the microcosms as shown in Figure 3.11. *Bacteroidetes* and *Betaproteobacteria* were abundant in the cellulose-amended microcosms, whereas *Firmicutes*-type *Sporolactobacillus* and *Clostridium* were abundant in glucose-amended microcosms. The *Verrucomicrobia*, *Gammaproteobacteria, Acidobacteria*, *Actinobacteria*, and *Alphaproteobacteria* divisions were prevalent in the native soil and in the control libraries.

At the genus level, *Bacteroidetes*, *Burkholderia*, and *Ralstonia* were abundant in the cellulose-amended microcosms, whereas *Sporolactobacillus* and *Clostridium* were abundant in glucose-amended microcosms (Figure 3.15, Table 3.6). Members of the *Rubrobactereaceae*, Grp4 *Acidobacteria*, *Rhizobiales*, and *Alphaproteobacteria* taxa were prevalent in the native soil and in the control libraries.

Notably, a number of unknown sequences were present in the libraries and were considered significant contributors to the community structure. Microbial communities from the control fraction (fraction 11 from day 54 ^{13}C-cellulose incubation), and from the day 14 ^{13}C-glucose incubation appeared to cluster near, and approximate the original tundra soil community.

3.4.7 Bacterial communities responsible for assimilating glucose in soil

The phylogenetic groups detected by DNA stable-isotope probing in this study are well-known inhabitants of soil ecosystems (Ceja-Navarro et al., 2010; Claus et al., 2006).

Major constituents of the original Arctic tundra sample include members of the *Alphaproteobacteria*, *Acidobacteria*, and *Actinobacteria*, which are commonly found in high abundance in Arctic tundra environments (Bartram et al., 2011; Wallenstein et al., 2007). The data suggests that assimilation of glucose was primarily performed by *Sporolactobacillus* and *Clostridium* spp. (Figure 3.15, Table 3.6).

Several previous studies have used stable-isotope probing techniques to study communities responsible for the metabolism of glucose in temperate agricultural soils (Padmanabhan et al., 2003, Schellenberger, Kolb, & Drake, 2010), a forest soil (Degelmann et al., 2009), and an anoxic fen (Hamberger et al., 2008). With the exception of the work performed by Padmanabhan and colleagues, the soil samples were incubated as soil slurries under anoxic conditions. Schellenberger and colleagues (2010) also examined bacterial communities retrieved from aerated soil slurries.

Padmanabhan et al. (2003) identified bacterial populations related to *Arthrobacter*, *Acinetobacter*, *Massilia*, *Flavobacterium*, *Pseudomonas*, and *Pedobacter* spp. as active consumers of glucose in a loam soil. Major phylogenetic groups involved in the assimilation of ^{13}C-labelled xylose and glucose in a German peat soil under aerobic conditions included *Acidobacteriaceae*, *Rhodospirallaceae*, *Enterobacteriaceae*, *Clostridium*, and *Sphingomonas* (Hamberger et al., 2008). Close relatives of the Enterobacteriaceae species *Rahnella aquatilis* and *Ewingella americana* were also identified by Degelmann and colleagues (2009) as anoxic glucose-degraders. Prominent taxa identified as aerobic degraders of cellulose by Schellenberger and colleagues (2010) included members of the phyla *Actinobacteria*, and members of the

families *Intrasporangiaceae* and *Micrococcaceae*. Anaerobic glucose metabolism was dominated by phylotypes affiliated with the saccharolytic cluster 1 of the *Clostridiaceae*.

The detection of members of the class *Clostridium* in previous ^{13}C –glucose incubation studies supports the present research. As shown in Figures 3.14 and 3.15, Clostridia were important for the structure of the bacterial communities and reached high levels of abundance in the 54-day glucose incubation. Members of Clostridia appear to dominate in anaerobic environments with glucose as a carbon source. Considering that the incubation was carried out under aerobic conditions, the activity of *Clostridium* was somewhat surprising, as aerobiosis in this class is unknown (Schellenberger et al., 2010). The presence and activity of *Clostridium* in the glucose incubations could be the result of the heterogeneity of soil environments. Components of soil such as minerals and organic compounds form aggregates that create microenvironments that support a broad diversity of microorganisms (Felske & Akkermans, 1998; Ranjard et al., 2000). Nunan and colleagues (2003) describe soil as a structured "mosaic" of bacterial patches of different densities based on soil architecture, nutrient availability, and soil porosity. Anoxic micro environments in aerated soils can be formed by moisture in soil, particularly after rainfall (Degelmann et al. 2009). Soil pores saturated with moisture in the microcosm soil would support obligate anaerobes. The persistence of obligately anaerobic Clostridia in oxic incubations was also reported by Schellenberger et al. (2010).

Members of the genus *Sporolactobacillus* are Gram-negative sporulating rods that can be either aerobic or microaerobic (Claus et al., 2006). They are common prokaryotic inhabitants of soil (Chen et al., 2005), and produce lactic acid from the fermentation of glucose and a variety of other carbohydrates (Kitahara & Suzuki, 1963). The ideal growth range for these organisms is

between 25-40°C, with slow growth reported at 15°C (Kitahara & Suzuki, 1963). Psychrophilic species of *Bacillus* have long been known to inhabit Arctic environments (Larkin & Stokes, 1966). Therefore the identification of *Sporolactobacillus* as a major glucose metabolizer in Arctic soil is supported by the literature. The endospores produced by Bacillus and Clostridia are highly resistant to temperature, UV, and radiation (Yang, 2010), which likely aids their survival in extreme environments such as Arctic tundra. The fact that the microorganisms that were dominant in glucose metabolism in this experiment were both spore-formers makes it difficult to determine if they are active community members in the original Arctic tundra environment. Long-range transmission of airborne microbiota has been previously shown (Bovallius et al., 1978), demonstrating a possible mechanism for spore transmission from other environments.

3.4.8 Bacterial communities responsible for assimilating cellulose in soil

In contrast to the predominance of particular taxa in the assimilation of glucose, metabolism of cellulose was performed by a diverse community. Prominent phyla identified by PCoA analysis included members of *Bacteroidetes* and the *Beta-* and *Gammaproteobacteria*. The phylum *Proteobacteria* is known to contain a diverse range of cellulolytic microorganisms. Previous researchers have identified cellulolytic phylotypes including *Burkholderia* (Belova et al., 2006), *Sorangium* (Hou et al., 2006), *Novosphingobium* (Landy et al., 2008), *Pseudomonas* (Dumova & Kruglov, 2009) and *Cellvibrio* (Pang et al., 2009). The diversity of bacterial taxa involved was expected due to the complex nature of cellulose as a substrate, and the variety of enzymes that are necessary for the complete degradation of the substrate (Lynd et al., 2002).

Recent studies by el Zahar Haichar et al. (2007), Bernard et al. (2007, 2009), Schellenberger et al. (2010), and Lee et al. (2011) have examined the bacterial communities responsible for cellulolysis in terrestrial soil environments. In their study of a French agricultural soil, el Zahar Haichar and colleagues (2007) identified organisms closely related to *Dyella*, *Mesorhizobium*, *Sphingomonas*, *Myxobacteria*, *Flavobacterium*, and *Streptomyces* as aerobic cellulose-degraders based on their presence in the heavy DNA from a DNA-SIP experiment. In another DNA-SIP study conducted in France by Bernard et al. (2007), *Beta*- and *Gammaproteobacteria* were predominant in the heavy fractions of DNA retrieved from soils amended with ^{13}C-labelled wheat, providing evidence for the involvement of these phyla in the degradation of plant biomass in aerobic environments. The same group performed a similar study on copper-contaminated soils, where they identified members of the *Gamma*- and *Deltaproteobacteria* as cellulose-degraders in pristine soils (Bernard et al., 2009). Similar results were obtained by Lee and colleagues (2011) in their study of cellulolytic communities in rice paddy soil. Bacteria assimilating ^{13}C-labelled rice callus based on DGGE band identities included members of the *Gammaproteobacteria*, *Bacilli*, *Flavobacteria*, *Chloroflexi*, *Sphingobacteria*, and *Clostridia*. Finally, the research by Schellenberger et al. (2010) identified taxa belonging to the *Bacteroidetes*, *Chloroflexi*, and *Planctomycetes* as aerobic cellulose degraders. In the same study, *Kineosporiaceae* (*Actinobacteria*), *Bacteroidetes*, and *Clostridiaceae* type organisms were shown to be active in cellulose metabolism under anoxic conditions (Schellenberger et al., 2010).

The results of these experiments are in accordance with the findings of the present study. *Bacteroidetes* and the *Beta*- and *Gammaproteobacteria* were important taxa responsible for the clustering of clone libraries from the cellulose microcosms in the PCoA analysis. Similar phyla

were identified as cellulose-degraders in the literature. Members of the *Gammaproteobacteria* in particular were consistently cited as active cellulolytic organisms in soil environments (Bernard et al., 2007, Bernard et al., 2009, Lee et al., 2011). Eight of the sequences identified from the DGGE excised bands were closely related to database sequences that were obtained from tundra soils, glaciers, and AntArctic ice. The data shown here are well supported by the literature, and thus the results obtained from this experiment provide further characterization of the consortia responsible for cellulolysis in soil environments.

Classification beyond the phylum level with the RDP Classifier (Wang et al., 2007) was not possible for some of the 16S rRNA gene clones that were sequenced using the RDP Classifier. Of the 374 sequences that were successful, 28 could not be classified beyond the phylum level even when the confidence threshold was relaxed to 50%. This number increased to 60 when the confidence threshold was raised to 90%. Clearly a large proportion of the 16S rRNA gene sequences achieved only weak association to the phylogenetic lineages currently represented in the RDP database.

Chapter 4: General discussion and further investigations

4.1 Characterization of tundra soil communities

The experiment described in the previous chapters involved the characterization of active microbial populations responsible for the metabolism of two carbohydrate sources. Chapter 2 provides a detailed description of the DNA stable-isotope probing technique, which was the foundation of the experiment described in Chapter 3. DNA stable-isotope probing is a recently developed technique that has been applied to terrestrial and aquatic habitats. The ability of this technique to link phylogenetic affiliation with physiological activity was useful for addressing the hypotheses of this experiment. The protocol outlined in Chapter 2 was adapted from a previous methodology in the Murrell lab (Neufeld et al., 2007b) and was published as an online instructional video with associated preliminary data hosted by the Journal of Visualized Experiments (JoVE; Dunford & Neufeld, 2010). This visualized protocol should prove useful considering that this technique can be difficult to conduct without a visual reference.

In Chapter 3, the communities of bacteria responsible for the assimilation of carbon from glucose and cellulose in an undisturbed Arctic tundra soil were characterized using DNA sequences extracted from DGGE polyacrylamide gels, 16S rRNA gene clone libraries, and principle co-ordinate analysis. I hypothesized that the communities characterized by these techniques would differ based on substrate amendment, and that a number of sequences would be difficult to classify due to the paucity of data for soil communities *in situ*. These hypotheses were confirmed by the experimental results. In the soil microcosms that were amended with glucose, the community composition of heavy DNA from the DNA-SIP gradient was

predominated in succession by organisms closely related to *Sporolactobacillus* and *Clostridium*. When cellulose was provided as a substrate, the communities present in the heavy fraction were more diverse and differed from the communities present in the microcosms amended with glucose. Taxa belonging to *Bacteroidetes*, and the *Gamma-* and *Betaproteobacteria* became more abundant, while the relative contribution of the *Alphaproteobacteria*, *Acidobacteria*, and *Actinobacteria* declined.

The diversity of microorganisms retrieved from sample microcosms reflects recent assertions that Arctic soils are functionally similar to soils in other biogeographic regions. Previously it was thought that the structural characteristics of these soils (i.e. the presence of permafrost) would exert a strong influence on bacterial community structure. Recently, several studies have indicated that soil pH is the most important factor affecting phylotype composition of bacterial communities (Chu et al., 2010; Lauber et al., 2009; Nicol et al., 2008). This is supported in the present study, as the communities identified here had strong similarities to previous studies that focused on temperate soils. The predominant cellulolytic taxa in particular were well represented in other DNA-SIP experiments described in the literature. In fact, one of the excised DGGE sequences was most closely related to a sequence from an uncultured *Actinobacteria* species identified in a soil DNA-SIP experiment performed by el Zahar Haichar et al. (2007). Excised DGGE bands typically displayed similarity to sequences obtained from glacier and tundra soil environments based on BLAST analysis (Table 3.6). Thus, the results obtained in this experiment are potentially representative of soil communities in general, and not just those occurring in the polar regions.

Microorganisms present in the soil microcosms appear to be evolutionarily divergent from the sequences obtained from well-characterized species. Molecular techniques such as DNA-SIP have only recently become available to researchers, and the continuous development of advanced sequencing platforms has only begun to shed light on uncultivated bacterial organisms. This study represents a proof-of-principle approach for the characterization of microbial taxa based on their assimilation of substrates of interest.

4.2 Limitations of the experimental design

The research presented in Chapter 3 was successful in identifying specific bacterial taxa associated with cellulose and glucose metabolism in a tundra soil from Resolute Bay, Nunavut, Canada. The results of this experiment correlate with information obtained by similar studies conducted with temperate soils (el Zahar Haichar et al., 2007, Bernard et al., 2007, Bernard et al., 2009, Schellenberger et al., 2010, Lee et al., 2011). This was a proof-of-principle study, which was intended to identify conditions suitable for DNA-SIP of Arctic tundra. However, several aspects of the design limited conclusions based on the data collected.

The incubation of the soil microcosms were conducted solely under aerobic conditions. In a similar experiment, Schellenberger and colleagues (2010) conducted parallel oxic/anoxic incubations of soil slurries amended with glucose or cellulose. The researchers found differences between the active communities in the microcosms based on the presence of oxygen in the microcosms. Incubating the microcosms under both conditions in the present experiment may have yielded further information regarding the metabolic capacities of the communities of bacteria in tundra soils. Nonetheless, the presence of obligately anaerobic *Clostridia* in the 16S

rRNA gene sequence libraries suggests that anoxic micro-environments were present in the microcosms. The existence of such environments could possibly compensate for the lack of anoxic microcosms, although my research did not test this claim specifically. Successful enrichment of microorganisms with stable isotope was inferred by following changes in DGGE community fingerprints generated from the heavy fraction recovered from the DNA-SIP centrifuge tubes. Other studies approximated substrate mineralization rates by tracking the evolution of ^{13}C-CO_2 in the headspace of the microcosms (el Zahar Haichar et al., 2007, Bernard et al., 2007). The measurement of headspace gas composition throughout the course of the incubation period would have been beneficial to this experiment, as a method for tracking isotope incorporation.

The ability of this study to characterize the communities responsible for cellulolysis is limited by the number of different substrates that were provided to the microbial communities in the soil microcosms. As discussed in Chapter 1, complete cellulolysis is the result of a complex network of enzymatic pathways. Cellulose is sequentially degraded into a number of smaller polymers including cellodextrins, cellobiose, and glucose (Zhang & Lynd, 2004). Pentose sugars including xylose and arabinose are constituents of hemicelluloses, another major constituent of plant biomass (Lavarack et al., 2002). In this study, only cellulose and glucose were provided as substrates. Therefore, it is difficult to fully characterize the organisms that became enriched in the cellulose microcosms. By providing different carbohydrates to the microcosms, the temporal progression of cellulolytic communities can be more accurately tracked, and the enzymatic capabilities of enriched microorganisms more clearly defined. Including DNA-SIP replicates would be helpful to assess the variability in a single time-point/single substrate incubation, and

future research would benefit from the inclusion of replication now that appropriate SIP incubation conditions have been established by this study.

The libraries that were the basis of the principle co-ordinate clustering were constructed using Sanger sequencing of clones containing gene sequences from the DNA-SIP fractions. This was a method for assessing community changes and for characterizing particular phylogenies. The libraries however were small, possibly limiting the phylogenetic diversity detectable in the heavy fractions. Some of the community members contributing to substrate metabolism may have been missed using this methodology, particularly if they were of low abundance. Recent advances in high-throughput sequencing technologies such as pyrosequencing (Sogin et al., 2006) and the Illumina platform (Bartram et al., 2011; Caporaso et al., 2010) allow for sequencing of microbial communities at a much greater level of coverage. A DNA-SIP experiment utilizing pyrosequencing has already been demonstrated by Pilloni et al. (2011) in their study of anaerobic toluene degraders. These sequencing technologies will benefit future DNA-SIP studies by increasing the sensitivity of the technique. Detection of rare members of the communities and subtle shifts in community structure will be more easily visualized by libraries containing thousands or millions of unique sequence reads.

The complexity and diversity of microbial communities makes them difficult to study using any one approach. The methods presented here demonstrate strategies for bypassing the high bacterial diversity present in soils so that phylotypes important to particular community functions can be identified. It is important to note that DNA-SIP is limited by substrate availability and the incorporation of labelled isotope into nucleic acids (Neufeld et al., 20007b). Each successful cellular division in the presence of 100% labelled substrate increases the stable-

isotope content of newly synthesized DNA strands by 50%. This limits DNA-SIP as a method for identifying previously unknown organisms as it 1) may fail to account for organisms with slow doubling times, 2) is impeded by any nutrient source that competes with the labelled substrate provided, and 3) can require substrate amendments at levels far greater than those experienced in a natural setting.

Variations on the stable-isotope probing technique have been developed in an effort to compensate for these limitations. Rather than relying on the incorporation of stable-isotopes into nucleic acids (which requires cell division), several researchers have analyzed isotopically enriched biomarkers instead. Phospholipid fatty acid stable-isotope probing (PLFA-SIP) is capable of achieving high resolution of organisms actively metabolizing substrates via comparison of cell membrane lipids (Boschker & Middelburg, 2002; Treonis et al., 2004). This negates the need for long incubation times and can be useful for identifying particular groups with unique membrane structures such as members of the *Eucarya* (fungi) and *Archaea*. A disadvantage is that PLFA-SIP relies on a very limited database of PLFA profiles based on known cultivated organisms. In terms of sensitivity, similar benefits are obtained through the use of RNA stable-isotope probing (RNA-SIP). RNA-SIP is very similar to DNA-SIP, but only requires the expression of isotopically labelled RNA molecules. These complementary techniques may be sensitive to metabolic activity earlier in the incubation than DNA-SIP (Neufeld et al., 2007a).

4.3 Considerations for future investigations

The research presented in this thesis is part of a proof-of-principle experiment. When complete, this project will represent the first combination of DNA-SIP and metagenomics as applied to a tundra soil community. As discussed in Chapter 1, one of the main interests in cellulolytic communities from an industrial perspective is the demand for novel cellulase enzymes with unique properties. Metagenomics is an ideal method for capturing functional enzymes from environmental samples (Schloss & Handelsman, 2003). In brief, metagenomics involves the construction of DNA libraries from microbial populations followed by screening for particular sequences or phenotypes of interest (Riesenfeld et al., 2004).

Metagenomics has previously been used to identify cellulolytic enzymes from environments including soil (Jiang et al., 2009), the termite hindgut (Warnecke et al., 2007), compost (Pang et al., 2009), bioreactor sludge (Jiang et al., 2010), the mammalian rumen (Ferrer et al., 2005), and enrichment cultures (Voget et al., 2003). These studies have been successful in identifying novel cellulase enzymes including endoglucanases and β-glucosidases, but have suffered from low hit rates of positive, cellulase-active clones (Duan & Feng, 2009). Metagenomics experiments targeting cellulases have been hindered by the diverse array of cellulase enzymes that are necessary for full microbial metabolism of the cellulose substrate. Sequence-based metagenomics approaches must target particular gene sequences that are conserved among the target populations. As cellulolysis is carried out by numerous different enzymes produced by members of the *Fungi* and *Bacteria*, it is unlikely that a sequence screen will capture the full complement of cellulolytic enzymes present in any given sample. Shotgun-sequencing approaches are also limited, as the ability to reconstruct metagenomic DNA

fragments decreases with community diversity (Chen & Murrell, 2010; Venter et al., 2004). Functional screening depends on the expression of heterologous genes taken up by host organisms such as *Escherichia coli*. Host expression is also problematic, as the host organism can be insufficient or biased at expressing the functional genes of interest (Duan & Feng, 2009). Functional screens can also require an inordinate number of clones, as the frequency of cellulolytic organisms can be low in comparison to the total population in natural environments (Ilmberger & Streit, 2010).

To compensate for these limitations, a method for reducing the complexity of the microbial communities is required to achieve maximal efficiency of the metagenomic technique (DeAngelis et al., 2010). This can be accomplished by enriching environmental samples with substrate. Particular taxa that possess enzymes capable of degrading the substrate would be enriched, increasing their representation in the overall population, and thus the likelihood that they would be represented in the constructed libraries. The gradient centrifugation step of the DNA stable-isotope probing technique provides an additional method for reducing community complexity. By enriching the sample with labelled substrate, microorganisms possessing the functional genes of interest can be isolated from the background community based on the buoyant density of their nucleic acids (Chen & Murrell, 2010, Pinnell et al., 2011). This should increase the frequency of positive clones, reducing the cost of discovering novel cellulolytic enzymes.

Heavy DNA from the DNA-SIP experiment described in Chapter 3 is currently undergoing metagenomic analysis by a collaborating researcher (Lee Pinnell). Another colleague (Yris Verastegui) is currently conducting a DNA-SIP and metagenomics study using a variety of

^{13}C-carbohydrates and assessment of SIP reproducibility with replication to further elucidate the role of particular taxa at varying stages of cellulose hydrolysis. Samples from several different soil ecosystems have been amended with labelled substrates including cellobiose, arabinose, xylose, glucose, and cellulose in this effort to survey functional community diversity and retrieve novel enzymes.

The results presented in this thesis advance the understanding of the diversity and composition of cellulolytic and glycolytic bacteria *in situ*. The work presented here provides the basis for a proof-of-principle coupled DNA-SIP and functional metagenomics experiment targeted towards retrieving novel cellulase enzymes directly from the environment. Tundra soil communities are at present poorly characterized, and the data collected during this experiment provide insight into the active cellulolytic communities in a tundra soil. Recent advances in next-generation sequencing platforms and functional metagenomics will continue to strengthen and apply this research by identifying genomes and enzymes of interest for industrial applications.

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